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Regulation of gene expression by the cAMP-Crp system in the soil bacterium *Pseudomonas putida*

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En memoria de mi abuela Alicia Corrales Campos (1938-2012), quien un día se durmió en su particular "País de las maravillas" para no volver jamás. QEPD

A Dios y a mi familia. Soy quien soy gracias a ustedes!

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¡Pura vida a todos!

Aleyo

GENERAL INDEX

Index of Figures ..... **iv**

Index of Tables ..... **vi**

Abbreviations ..... **vii**

Abstract ..... **xi**

Resumen ..... **xiii**

I. Introduction **1**

1 Global regulation of gene expression 3

2 The archetypal cAMP-Crp system of *E. coli* 5

2.1 The cAMP-receptor protein (Crp) 5

2.2 Carbon Catabolite Repression mediated by cAMP-Crp in *E. coli* 8

2.3 Transcription activation at simple Crp-dependent promoters 11

3 Adenylate cyclase (CyaA) 14

4 cAMP phosphodiesterase (cAMP PDE) 15

5 Virulence factor regulator (Vfr) of *Pseudomonas aeruginosa* 16

6 cAMP-Crp system in *Pseudomonas putida* 17

7 The issue at stake 19

II. Objectives **21**

III. Materials and Methods **25**

1 Culture conditions and media 27

2 Bacterial strains 27

3 Plasmids 29

4 Recombinant DNA techniques 31

5 Protein techniques 34

6 Construction of Δ*crp* and Δ*cya*A strains of *P. putida* 35

7 *crp* and *cya*A complementation assays 35

7.1 Construction of single Δ*crp* and double Δ*crp* Δ*cya*A strains of *E. coli* 35

7.2 Design of expression vectors for *crp* and *cya*A genes of *E. coli* and *P. putida* 36

8 *Dictyostelium discoideum* cAMP chemotaxis biosensor 37

9 Quantification of cAMP produced by *E. coli* and *P. putida* 38

10 Protein purification 39
10.1 Production and purification of MBP-Crp*P. putida* fusion protein 39
10.2 Cleavage of MBP-Crp and purification of Crp*P. putida* 40
10.3 Production and purification of apo-Crp*P. putida* 40
11 Analytical ultracentrifugation of Crp*P. putida* 41
12 Partial proteolysis of Crp*P. putida* 42
13 Isothermal titration calorimetry (ITC) 42
14 Electrophoretic mobility shift assays (EMSA) 43
15 *In vitro* transcription assays (IVT) 44
16 β-galactosidase assays 45
16.1 Construction of plasmids encoding *lac*Z transcriptional and translational fusions 45
16.2 Measurements of the β-galactosidase activity 45
17 RNA-seq transcriptome analysis of *crp* and *cya*A mutants in *P. putida* 46
17.1 Culture conditions, RNA isolation and rRNA removal 46
17.2 RNA-Seq using the Illumina Genome Analyzer 47
17.3 Bioinformatic analysis 48
17.4 Estimation of Differential Expression 49

IV. Results 51

Chapter I. Genetic and phenotypic characterization of the components involved in the cAMP-Crp system of *Pseudomonas putida* 53

1 The cAMP-Crp system of *Pseudomonas putida* and its components. 54
2 Functional characterization of *P. putida crp* and *cya*A gene products 57
2.1 The *crp* gene of *P. putida* and its encoded transcriptional regulator 57
2.2 *In vivo* functional complementation of Crp*E. coli* by Crp*P.putida* 59
2.3 *In vivo* functional complementation of CyaA*E. coli* by CyaA*P. putida* 60
2.4 *In vivo* complementation with both Crp and CyaA of *P. putida* and *E. coli* 62
3 Concentrations of cyclic AMP in *P. putida* 64
3.1 Detection of cAMP by the slime mold *Dictyostelium discoideum* 64
3.2 Quantification of the intracellular and extracellular levels of cAMP 65
4 The *cya*A gene is transcribed, but it is not efficiently translated in *P. putida* 66

5 The production of Crp$_{P.\ putida}$ is slightly increased in cells growing on glucose.....68

Chapter II. Biochemical characterization of *P. putida* Crp: evidence evidence of an unusually high affinity for cAMP 71

1 Purification of Crp$_{P.putida}$ as an MBP-fusion protein.....72

2 Crp$_{P.putida}$ is a dimeric protein that undergoes a conformational change upon cAMP binding.....74

3 Crp$_{P.putida}$ displays an extraordinary high affinity for cAMP 76

4 Crp$_{P.putida}$ binds one cAMP molecule per protein dimer.....78

5 Crp$_{P.\ putida}$ activates the transcription machinery of *P. putida* in a class I Crp-dependent promoter.....81

Chapter III. Detection of genes directly or indirectly regulated by the components of the cAMP-Crp system in *P. putida* 83

1 RNA-seq transcriptome analysis of *crp* and *cya*A mutants of *P. putida* 84

V. Discussion 89

1 The cAMP-Crp system of *Pseudomonas putida* 91

2 *P. putida* CyaA produces very low levels of cAMP 92

3 CyaA and Crp levels are slightly increased in cells growing on glucose and are not autoregulated by cAMP-Crp 94

4 Crp$_{P.putida}$ is a dimeric protein that undergoes a conformational change upon cAMP binding.....95

5 Crp$_{P.putida}$ displays an extraordinary high affinity for cAMP 96

6 A negative cooperativity model of cyclic AMP binding explains the 1:1 Crp$_{P.\ putida}$-dimer:cAMP stoichiometry 97

7 Crp$_{P.\ putida}$ activates the transcription machinery of *P. putida* and *E. coli* 98

8 RNA-seq transcriptome analysis of Δ*crp* and Δ*cya*A mutants of *P. putida*.....98

9 cAMP-Crp system of gene regulation: an example of *Regulatory exaptation*.....100

VI. Conclusions..... 103

VII. References 107

VIII. Annexes..... 127

INDEX OF FIGURES

Fig. 1. Tridimensional structure of the cAMP-Crp complex of *E. coli* bound to DNA 6

Fig. 2. Mechanism of allosteric control of *E. coli* Crp (CAP) 7

Fig. 3. Classical model of the Carbon Catabolite Repression (CCR) of carbohydrates in *E. coli* 10

Fig. 4. Mechanisms of transcription activation at Crp-dependent promoters 13

Fig. 5. Pipeline of the RNA-seq protocol for transcriptome analysis using the *Illumina* sequencing platform 47

Fig. 6. Components of the cAMP-Crp system of *P. putida* KT2440 55

Fig. 7. Growth curves of cAMP-Crp system mutants on three different carbon sources 56

Fig. 8. Comparison between the Crp homologues in *E. coli*, *P. aeruginosa* and *P. putida* 58

Fig. 9. Complementation analysis of *P. putida crp* and *cyaA* genes in *E. coli* 60

Fig. 10. Detection of cAMP production mediated by the CyaA product of *P. putida* 61

Fig. 11. Complementation of the Δ*cya*A Δ*crp* double mutant of *E. coli* with combinations of plasmids encoding *crp* and *cya*A genes of *E. coli* and *P. putida* 63

Fig. 12. *D. discoideum* cAMP chemotaxis test *in vivo* 64

Fig. 13. Analysis of cAMP production by *E. coli* and *P. putida* 66

Fig. 14. Transcription and translation of the *cya*A gene of *P. putida* 67

Fig. 15. β-galactosidase activity of a translational *crp'-'lac*Z gene fusion 69

Fig. 16. Expression and purification of MBP-Crp$_{P.putida}$ in *E. coli* 72

Fig. 17. Cleavage of MBP-Crp$_{P. putida}$ and purification of MBP-less Crp$_{P. putida}$ 73

Fig. 18. Oligomerization state of *P. putida* Crp 74

Fig. 19. Partial proteolysis of Crp$_{P. putida}$ 75

Fig. 20. Isothermal titration microcalorimetry of MBP-Crp$_{P. putida}$ interacting with putative effector molecules 77

Fig. 21. Isothermal titration microcalorimetry of apo-Crp$_{P. putida}$ interacting with cAMP and cGMP 79

Fig. 22. DNA recognition and transcription activation of the P*lac* promoter mediated by Crp$_{P. putida}$ 81

Fig. 23. Differential expression pattern of genes in the Δ*crp* mutant of *P. putida* compared with reference KT2440 wild-type strain 85

Fig. 24. Differential expression pattern of genes in the Δ*cya*A mutant of *P. putida* compared with reference KT2440 wild-type strain 86
Fig. 25. Venn diagrams illustrating the genes that are co-regulated by Crp and CyaA in *P. putida* 87

INDEX OF TABLES

Table 1. Description of the Bacterial strains used in this study.....28

Table 2. Plasmids employed in this work.....29

Table 3. Oligonucleotides designed in this Thesis for PCR amplification.....33

Table 4. Thermodynamic parameters obtained from titration of either MBP-Crp$_{P.\ putida}$ fusion protein or apo-Crp$_{P.\ putida}$ with putative effectors. 78

Table 5. List of upregulated genes in *P. putida* KT2440 Δ*crp vs*. wild-type KT2440 strain in M9-glucose cultures.....129

Table 6. List of downregulated genes in *P. putida* KT2440 Δ*crp vs*. wild-type KT2440 strain in M9-glucose cultures.....131

Table 7. List of upregulated genes in *P. putida* KT2440 Δ*cya*A *vs*. wild-type KT2440 strain in M9-glucose cultures.....132

Table 8. List of downregulated genes in *P. putida* KT2440 Δ*cya*A *vs*. wild-type KT2440 strain in M9-glucose cultures. 134

ABBREVIATIONS

Å	Angstrom
aa	Amino acid
AC	Adenylate cyclase
AMP	Adenosine monophosphate
Ap	Ampicillin
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
C-source	Carbon Source
cAMP	Cyclic AMP or 3'-5'-cyclic adenosine monophosphate
CBD	cAMP binding domain
CCR	Carbon catabolite repression
cDNA	Complementary DNA
CDS	Coding sequence
cGMP	Cyclic GMP or 3',5'-cyclic guanosine monophosphate
cm	Centimeter
Cm	Chloramphenicol
cNMP	Cyclic nucleotide monophosphate
CTP	Cytidine triphosphate
Da	Dalton
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleic triphosphate
DTT	Dithiothreitol
ED	Entner-Doudoroff
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
FDR	False Discovery Rate
FMN	Flavin mononucleotide
GC	Guanylate cyclase

Glc	Glucose
GTP	Guanosine triphosphate
HPLC-ESI-MS	High Performance Liquid Chromatography – electrospray tandem mass spectrometry
HTH	Helix-turn-helix
IPTG	Isopropyl β-D-thiogalactopyranoside
ITC	Isothermal Titration Microcalorimetry
IVT	*In vitro* transcription
K_A	Affinity constant
kb	1000 base pairs
kcal	Kilocalories
K_D	Dissociation constant
kDA	Kilodalton
Km	Kanamycin
Lac	Lactose
LB	Luria-Bertani médium
M	Molar
Mal	Maltose
MBP	Maltose binding protein
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
MWCO	Molecular weight cut-off
N-source	Nitrogen Source
ncRNA	Non-coding RNA
ng	Nanogram
nm	Nanometers
nM	Nanomolar
OD	Optical density

ONPG	*ortho*-Nitrophenyl-β-galactoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
pmol	Picomol
PTS	Phosphoenolpyruvate-carbohydrate phosphotransferase system
RF	Uncharacterized regulatory factor necessary for AC activation
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNase	Ribonuclease
RPKM	Reads Per Kilobase per Million mapped reads
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulfate
SELEX	Systematic Evolution of Ligands by Exponential enrichment
T3SS	Type III secretion system
Tc	Tetracycline
TCA	Tricarboxylic acid
TF	Transcription factor
tRNA	Transfer RNA
U	Units
UTP	Uridine triphosphate
UTR	Untranslated region
UV	Ultraviolet
wt	Wild-type
α	Alpha subunit of RNA polymerase
Δ*G*	Gibbs free energy change

Δ*H*	Enthalpy change
Δ*S*	Entropy change
µcal	Microcalories
µCi	Microcurie
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
σ	Sigma factor of RNA polymerase

ABSTRACT

The genome of the soil bacterium *Pseudomonas putida* KT2440 encodes orthologs of genes *crp* (cAMP receptor protein), *cya*A (adenylate cyclase) and *cpd*A (cAMP phosphodiesterase) of *Escherichia coli*. The $crp_{P.putida}$ gene restored the lack of maltose consumption of a *crp* mutant of *E. coli,* but not in a double *crp/cyaA* strain, suggesting that the ability to regulate such functions required cAMP. On the other hand, the $cyaA_{P.\ putida}$ gene was transcribed *in vivo* but failed to complement the metabolic capacities of an equivalent *cya*A mutant of *E. coli*. Yet, generation of cAMP by $CyaA_{P.\ putida}$ could be verified by expressing the $cyaA_{P.\ putida}$ gene in a hypersensitive *E. coli* strain, thereby indicating that cAMP was poorly synthetized by the adenylate cyclase of *P. putida*. The low levels of cAMP formed by *P. putida* cells were further confirmed either chemically or with a *Dictyostelium* biosensor *in vivo*. Using transcriptional and translational fusions of $cyaA_{P.\ putida}$ promoter with *lac*Z, we demonstrated that such a low cAMP levels stem from the fact that the adenylate cyclase is transcribed but it is not efficiently translated. Also, the Crp regulator of *P. putida* was purified and subjected to a battery of *in vitro* assays aimed at determining its principal physicochemical properties. Analytical ultracentrifugation indicated effector-free $Crp_{P.\ putida}$ to be a dimer in solution that undergoes a significant change in the presence of cAMP. Such a conformational transition was confirmed by limited proteolysis of the protein in the absence or presence of the effector. Thermodynamic parameters calculated by isothermal titration calorimetry revealed a tight 1:1 cAMP:$Crp_{P.\ putida}$ dimer association with an apparent K_D of 45.0 ± 3.4 nM, i.e. much greater affinity than that reported for the *E. coli*'s counterpart. The regulator also bound cGMP, but with a K_D ~100 fold lower. An *in vitro* transcription system was then set up with purified *P. putida*'s RNA polymerase for examining the preservation of the correct protein–protein architecture that makes Crp to activate target promoters. These results, along with cognate gel shift assays indicated that all basic features of the reference $Crp_{E.\ coli}$ are kept in *P. putida*. Finally, RNA-seq transcriptome analysis demonstrated that cAMP-$Crp_{P.\ putida}$ is not related with the regulation of metabolic functions, but it rather controls factors for the cellular response to environmental stress and the expression of components of the bacterial membrane to shape the cell envelope in response to harmful conditions. This poor metabolic profile of the cAMP-Crp system of *P. putida* when compared with *E. coli* exposes a case of regulatory exaptation i.e. the process through which a property evolved for a particular function is co-opted for a new use.

RESUMEN

El genoma de la bacteria del suelo *Pseudomonas putida* KT2440 codifica ortólogos de los genes *crp* (proteína receptora de cAMP), *cya*A (adenilato ciclasa) y *cpd*A (fosfodiesterasa de cAMP) presentes en *Escherichia coli*. En este trabajo demostramos que el gene $crp_{P.putida}$ logra restaurar la incapacidad para consumir maltosa en un mutante *crp* de *E. coli*, pero no así en un doble mutante *crp/cya*A de esta bacteria, sugiriendo que la habilidad para regular las funciones dependientes de $Crp_{P.putida}$ requieren cAMP. Por otra parte, mostramos que el gene $cyaA_{P.\ putida}$ se transcribe *in vivo*, pero es incapaz de complementar estas capacidades metabólicas en un mutante equivalente del gene *cya*A en *E. coli*. No obstante, la producción de cAMP por la proteína $CyaA_{P.putida}$ se pudo verificar posteriormente al expresar el gene $cyaA_{P.\ putida}$ en una cepa de *E. coli* hipersensible a bajas concentraciones de cAMP, indicando que la síntesis de cAMP en *P. putida* es muy baja. Estos niveles tan bajos del cAMP fueron confirmados posteriormente tanto de forma química como por un biosensor *in vivo* basado en *Dictyostelium*. Mediante fusiones transcripcionales y traduccionales del promotor del gene $cyaA_{P.\ putida}$ con *lac*Z, demostramos que la baja producción de cAMP en *P. putida* radica en que el gen de la adenilato ciclasa se transcribe eficientemente, pero su nivel de traducción es muy bajo. Adicionalmente, la proteína Crp de *P. putida* se purificó y se sometió a un conjunto de ensayos *in vitro* para determinar sus principales propiedades físico-químicas. Mediante ultracentrifugación analítica, mostramos que la proteína $Crp_{P.\ putida}$ forma dímeros en solución, los cuales sufren un importante cambio conformacional al interaccionar con el cAMP. Este cambio se demostró asimismo utilizando ensayos de proteólisis parcial en ausencia o presencia de este efector. Por medio de ensayos de microcalorimetría, se calcularon los parámetros termodinámicos de la unión de con el cAMP. Esta interacción resultó llevarse a cabo con una estequiometría de 1 molécula cAMP: 1 dímero $Crp_{P.putida}$ y se reveló que la unión es especialmente fuerte, presentando un valor aparente de la K_D de 45.0 ± 3.4 nM, es decir, una afinidad mucho mayor que la conocida para la proteína homóloga de *E. coli*. $Crp_{P.putida}$ también unió cGMP pero con una K_D cerca de 100 veces menor. Posteriormente utilizamos un sistema de transcripción *in vitro* empleando la ARN polimerasa purificada de *P. putida* para examinar la conservación de las interacciones proteína-proteína que permiten la activación de los promotores dependientes de Crp. Estos experimentos, junto con sus respectivos ensayos de retardo de la movilidad electroforética, indicaron que todas las características básicas de la $Crp_{E.coli}$ también se encuentran presentes en la Crp de *P. putida*. Finalmente, análisis transcriptómicos utilizando ultra-secuenciación profunda (ARN-seq) demostraron que el sistema cAMP-Crp no regula

funciones metabólicas en *P. putida*, sino que por el contrario parece controlar factores relacionados con la respuesta celular a estrés ambiental y la expresión de componentes de la envuelta celular en tales condiciones estresantes. Este pobre perfil metabólico del sistema cAMP-Crp en *P. putida* en comparación con *E. coli* expone un claro caso de *exaptación reguladora*. Este es el proceso por el que un componente biológico que emerge por primera vez para cumplir una función particular es reclutado para desempeñar un nuevo uso sin cambiar su estructura.

I. Introduction

1 Global regulation of gene expression

The wide diversity of microorganisms in nature live in a variety of environments subject to rapid changes in the availability of the carbon and nitrogen compounds necessary to provide energy and building blocks for the synthesis of cell material. Their survival depends on their ability to adapt to these changes by regulating the expression of genes coding for enzymes and transport proteins required for growth in the altered environment (Magasanik, 2000). This gene expression is a highly complex process that comprises many steps, each with elaborate regulation. In bacteria, transcription initiation is the major step of regulation, although mRNA abundance is also controlled at the step of transcription elongation and termination. Degradation of mRNA is also subject to control and, furthermore, increasing data indicate the involvement of translational control of mRNA through various mechanisms, including the interference of mRNA translation by regulatory RNAs and proteins (Ishihama, 2010).

The promoters of bacterial genes for such environmental signals are controlled by numerous proteins through direct protein-DNA or protein-protein interactions that either up- or downregulate the transcription by the multi-subunit RNA polymerase (RNAP; Perez-Rueda & Collado-Vides, 2000; Browning & Busby, 2004; Ishihama, 2010). These proteins include sigma (σ) factors (and their anti-sigma-factor counterparts), which associate with the core of the RNAP to initiate the transcription, and the transcriptional factors (TFs) that target sequences frequently found near promoter regions (Cases & de Lorenzo, 2005; Wosten, 1998). Such factors also include nucleoid-associated proteins, typically the integration host factor (IHF), the factor for inversion stimulation (FIS) and the regulator for leucine LRP (Cases & de Lorenzo, 2005); as well as more generic events like changes in chromosomal supercoiling (Hatfield & Benham, 2002) or the intracellular concentration of osmolytes like potassium glutamate, glycine betaine, L-proline and trehalose (Lee & Gralla, 2004). Additionally, the promoter architecture and the conservation of the consensus recognition sequences by RNAP (-10 and -35 promoter elements for example), acts as a powerful regulator in the gene expression and in the distribution of RNA polymerase amongst the different transcription units (Browning & Busby, 2004).

The genome of the model prokaryote *Escherichia coli* encodes for about 300 types of TFs (Perez-Rueda & Collado-Vides, 2000; Madan Babu & Teichmann, 2003). The regulatory function is not known at all for approximately a third of them (Ishihama, 2010). When bound to specific DNA target sites near promoters, these proteins recruit the RNAP to the promoter region by interacting directly with one or more subunits of the RNAP complex, in order to activate the transcription. However, some TFs bind to promoter sequences to repress the transcription by overlapping core promoter elements -steric hindrance-, by forming DNA loops that avoid the interaction of RNAP with the intervening promoter -repression by looping- and by interacting directly with an activator, preventing its function -modulation of an activator- (reviewed in Browning & Busby, 2004). Some of these transcriptional factors control only few genes (local regulators), whereas others control simultaneously a large number of genes and therefore, many metabolic conditions. The last set of factors are known as global regulators and include Crp, FNR, IHF, Fis, ArcA, NarL, Lrp, RutR, Cra and Dan (Martinez-Antonio & Collado-Vides, 2003; Browning & Busby, 2004; Cases & de Lorenzo, 2005; Ishihama, 2010). In fact, the first seven TFs described above are sufficient to directly modulating the expression of 51% of genes in *E. coli* (Martinez-Antonio & Collado-Vides, 2003), thereby exhibiting pleiotropic phenotypes (Ishihama, 2010).

To respond immediately to the environmental signals, the function of the TFs must be regulated either by controlling their activity or their expression. There are at least four different mechanisms to achieve this (reviewed by Browning & Busby, 2004): [i] the affinity of some regulators could be modulated by covalent modification like phosphorylation mediated by TF-cognate sensor kinases. This is the case of NarL, which activity is controlled in response to extracellular nitrate and nitrite by the sensor kinases NarX and NarQ (Stock *et al.*, 2000); [ii] the intracellular concentration of some regulators also can control their activity, like the SoxS regulator of oxidative stress in *E. coli*, or the XylS regulator of the *meta*-cleavage pathway for m-xylene/toluene degradation encoded in the TOL plasmid of *P. putida* mt-2 (Gonzalez-Perez *et al.*, 2004). In these cases the concentration of the TF is controlled either by regulation of expression or by proteolysis; [iii] a less common mechanism for regulating the effective concentration (and therefore, the activity) of a TF is sequestration by another regulatory protein to which it binds. This is the case of Mlc global repressor, which is sequestered by the $IIBC^{Glc}$ complex of the

phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS; see below) in presence of glucose (Tanaka *et al.*, 2000); [iv] finally, one frequent mechanism to modulate the DNA-binding affinity in TFs is by interaction with small ligands, the concentrations of which fluctuate in response to nutrient availability or stress. This is the case of the Lac repressor, which DNA-binding affinity is reduced by allolactose. Amongst these small signalling molecules, the cyclic nucleotides count among the most widespread throughout the entire Tree of Life (Gomelsky, 2011), the proteins whose biological activity is regulated upon their binding diversify enormously (Shabb & Corbin, 1992). Virtually all bacterial genomes have been found to encode proteins that bind and respond to 3'-5'-cyclic adenosine monophosphate (cAMP), the prototypical of these being the transcriptional regulator of *E. coli*, first designated as the catabolite activator protein (CAP) and later named with the more unbiased name of cAMP-receptor protein or Crp (Busby & Ebright, 1999).

2 The archetypal cAMP-Crp system of *E. coli*

2.1 The cAMP-receptor protein (Crp)

The cAMP-receptor protein of *E. coli* (Crp or CAP) was the first transcriptional activator ever identified because of its positive role in expression of the *lac* operon (Ullmann & Monod, 1968; Zubay *et al.*, 1970). This protein belongs to the Crp-FNR superfamily of transcriptional regulators that include nearly 370 DNA-binding members, which predominantly function as positive transcription factors (Korner *et al.*, 2003). Crp-FNR proteins are characterized by a C-terminally located helix-turn-helix (HTH) structural motif consisting of two α-helices joined by a turn, which fits into the major groove of DNA. In addition to the HTH motif, Crp-FNR regulators have a large nucleotide-binding domain that extends from the N-terminus over roughly 170 residues (Korner *et al.*, 2003). Early in the study of Crp, it became clear that this protein had the ability of occupying a target DNA sequence in the proximity of the RNAP binding site of P*lac* depending on the binding to cAMP as co-activator. Later work has shown that Crp is in fact a homodimeric protein, each 209 amino acids monomer folded into two distinct structural domains (Fig. 1). The larger amino-terminal cAMP-binding domain (CBD; comprising residues 1-136) is responsible for the interaction with the allosteric effector cyclic AMP. It also contains the

long C α-helix, which is instrumental in protein dimerization. The carboxy-terminal DNA-binding domain is smaller (DBD; comprising residues 140-209) and mediates the interaction of Crp with DNA through a helix-turn-helix DNA-binding motif (McKay & Steitz, 1981; Kolb *et al.*, 1993; Busby & Ebright, 1999). Both domains are separated by a short hinge region (residues 136-139), which is involved in the regulation of the allosteric conformational change that occurs upon cAMP binding to Crp, including subunit realignment and domain rearrangement (Gao *et al.*, 2012).

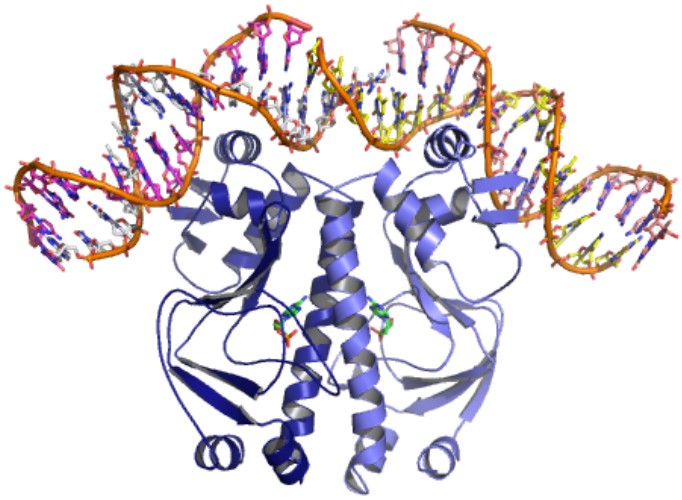


Fig. 1. Tridimensional structure of the cAMP-Crp complex of *E. coli* bound to DNA. The cAMP-bound Crp-dimer interacts with the major groves of a DNA sequence similar to consensus (5'-GCGAAAAGTGTGACATATGTCACTTTTCG-3') by direct contact of the F-helices located in the C-terminal DNA-binding domain of both subunits. Note the localization of the cAMP allosteric effector in the nucleotide-binding pocket situated in the N-terminal domain of Crp-dimer. The crystallographic structure at 2.5 Å was obtained from PDB accession ID 1J59 DNA (Parkinson *et al.*, 1996).

The *E. coli* cAMP-Crp complex of *E. coli* seems to target sequences best containing 5'TGTGA-6N-TCACA3' (extended: 5'AAA**TGTGA**TCTAGA**TCACA**TTT3') although this optimal configuration hardly appears as such because permanent occupation of the DNA site may not be biologically useful (Ishihama, 2010; Shimada *et al.*, 2011). Target sequences are often located at varying distances upstream the transcription start site of the RNAP (Busby & Ebright, 1999). Depending on this distance, the mode of transcription activation by Crp is different (this issue will be revisited later). However, Crp can also repress the transcription of some promoters by either preventing the RNAP from binding

to the promoter DNA (usually the Crp-binding site overlaps the -10 or -35 sequences of the promoter), or by blocking transcription from another upstream promoter (Zheng *et al.*, 2004).

Recently, the cAMP-mediated allosteric transition of Crp from the inactive to the active DNA-binding conformation was proposed by Won *et al.* (2009) and finally demonstrated by Popovych and colleagues (2009). The basic feature of this mechanism is a bistable coiled-coil structure formed in the intersubunit of the C-helix/C'-helix (where ' denotes the second Crp subunit) determined by the interaction with cAMP (Fig. 2). The cyclic nucleotide adenine base directly interacts with residues Thr-127 and Ser-128, causing both residues to relocate and to reorientate. This generates a coil-to-helix transition from Val-126 to Phe-136, resulting in the extension of the C-helix by three turns of helix in each Crp-subunit. The coil-to-helix transition results in the rotation of the DNA-binding domain by ~60° and its translation by ~7 Å. This rotation and translation places the DNA-recognition helices (F-helices) of both subunits in the correct orientation and in the correct position to interact with the successive DNA major grooves, making extensive contacts with DNA base pairs and the sugar phosphate backbone (Parkinson *et al.*, 1996; Popovych *et al.*, 2009). Interaction of Crp F-helices with DNA leads to an extensive bend of the

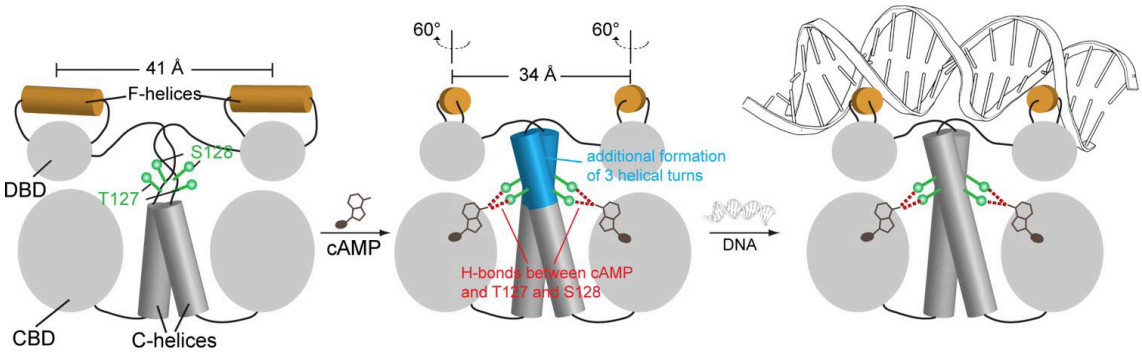


Fig. 2. Mechanism of allosteric control of *E. coli* Crp (CAP). Schematic models of Crp in the 3 structurally characterized states: apo-Crp, Crp-cAMP2, and Crp-cAMP2-DNA. The mechanism of allosteric control involves the binding of cAMP to the CBD of Crp, making contacts with Thr-127 and Ser-128. These contacts induce a coil-to-helix transition that extends the C-helix, and the intersubunit C-helix/C'-helix coiled coil by 3 helical turns (represented in cyan in the Crp-cAMP2 model). This coil-to-helix transition results in rotation and translation of the DBD of the Crp dimer. This conformational change places the F-helices (DNA-recognition helices) of the Crp dimer in the correct orientation to interact with DNA. (Popovych *et al.*, 2009).

DNA double helix by about ~80°. The orientation of this Crp-induced DNA bend is such that the DNA wraps towards and around both sides of the homodimer (Parkinson *et al.*, 1996; Chen *et al.*, 2001; Busby & Ebright, 1999). Thus, DNA-bending facilitates the interaction between Crp and the RNAP to promote transcription activation in some Crp-dependent promoters (see below).

Although Crp is an abundant protein in *E. coli* (Anderson *et al.*, 1971) it seems that its regulatory scope has specialized in controlling metabolic functions in this bacterium (Kolb *et al.*, 1993). Up to the present, about 200 target promoters have been recorded in RegulonDB database (http://regulondb.ccg.unam.mx/) to be regulated in some extent by Crp, many of them encoding transporters and catabolic enzymes for the metabolization of carbon sources (Gama-Castro *et al.*, 2011; Shimada *et al.*, 2011). Moreover, genome-SELEX screening of Crp-regulated promoters identify at least 183 novel operons in *E. coli*, which added to the near 200 hitherto known targets, encompass approximately 380 promoters as the regulation targets for cAMP-CRP (Shimada *et al.*, 2011). The novel target genes identified by Shimada and colleagues are involved in metabolic processes of the cell, such as selective transport of carbon sources, the glycolysis-gluconeogenesis switching to the metabolisms downstream of glycolysis, including tricarboxylic acid (TCA) cycle, pyruvate dehydrogenase (PDH) pathway and aerobic respiration (Shimada *et al.*, 2011). The regulation of such metabolic traits, converts Crp in one of the major players in the global pathway of Carbon Catabolite Repression (CCR) in *E. coli* (Stulke & Hillen, 1999; Deutscher, 2008; Gorke & Stulke, 2008; Shimada *et al.*, 2011). Apart of being one key mediator of CCR, this factor ultimately orchestrates the distribution of carbon fluxes into respiratory and fermentative metabolisms (Haverkorn van Rijsewijk *et al.*, 2011).

2.2 Carbon Catabolite Repression mediated by cAMP-Crp in *E. coli*

Bacteria have developed sophisticated mechanisms to adapt to environmental changes, for example the carbon availability. These microorganisms are able to distinguish between a *preferred* (i.e. rapidly metabolizable or energetically favourable) carbon source when they are exposed to more than one of these nutrients. To achieve this, the bacteria synthetize only the enzymes necessary for utilizing the preferentially consumed source of carbon (Deutscher, 2008; Stulke & Hillen, 1999). This regulatory phenomenon is known as Carbon Catabolite Repression, and is defined as the mechanism by which the expression of

functions for the use of secondary carbon sources and the activities of the corresponding enzymes are reduced in the presence of the preferred carbon source (Cases & de Lorenzo, 1998; Gorke & Stulke, 2008). CCR is widely distributed amongst bacteria, as it represents an important mechanism for competition in natural environments, where selection of the preferred carbon source represents an adaptive advantage for the organisms (Ullmann, 1996; Gorke & Stulke, 2008). In some pathogenic bacteria, for example, CCR has a crucial role in the expression of virulence genes, which often enable bacteria to access new sources of nutrients (Gorke & Stulke, 2008). This phenomenon plays also a major role in the genetic manipulation of bacteria for biotechnological or environmental applications, because the main bottleneck in the expression of heterologous genes or factors for these purposes is the general downregulation of promoter activity caused by environmental and metabolic conditions (Cases & de Lorenzo, 1998).

The first quantitative analysis of CCR was observed by Jacques Monod in 1942. He grew bacterial cultures in the presence of two carbohydrates instead of a single one, and depending on the nature of the two carbohydrates (i.e. one preferred carbon source as glucose and a secondary carbon source such as lactose), the growth curve exhibited two successive growth cycles separated by a period of lag, describing a diauxic growth curve (Monod, 1942). However, it was not until 1965 that cAMP was identified as an antagonist of the repressing effect of glucose over alternative carbon sources in *E. coli* cells (Ullmann & Monod, 1968). Later, Zubay and colleagues (1970) discovered the protein factor which acts in conjunction with cAMP, called catabolite gene activator protein (CAP or Crp).

In *E. coli*, the major players in the global pathway of CCR are the above-described Crp (together with its cognate signal cAMP) and the PTS system (Gorke & Stulke, 2008). The PTS is a multiprotein phosphorelay system that catalyses the uptake of numerous carbohydrates and their conversion in their respective phosphoesters during transport. Thus, it couples transport and phosphorylation of carbohydrates by the action of the protein complex formed by two general cytoplasmic components, Enzyme I (EI, encoded by *pts*I gene) and HPr (encoded by *pts*H gene), and the complex formed by the Enzyme II (EII; Deutscher *et al.*, 2006).

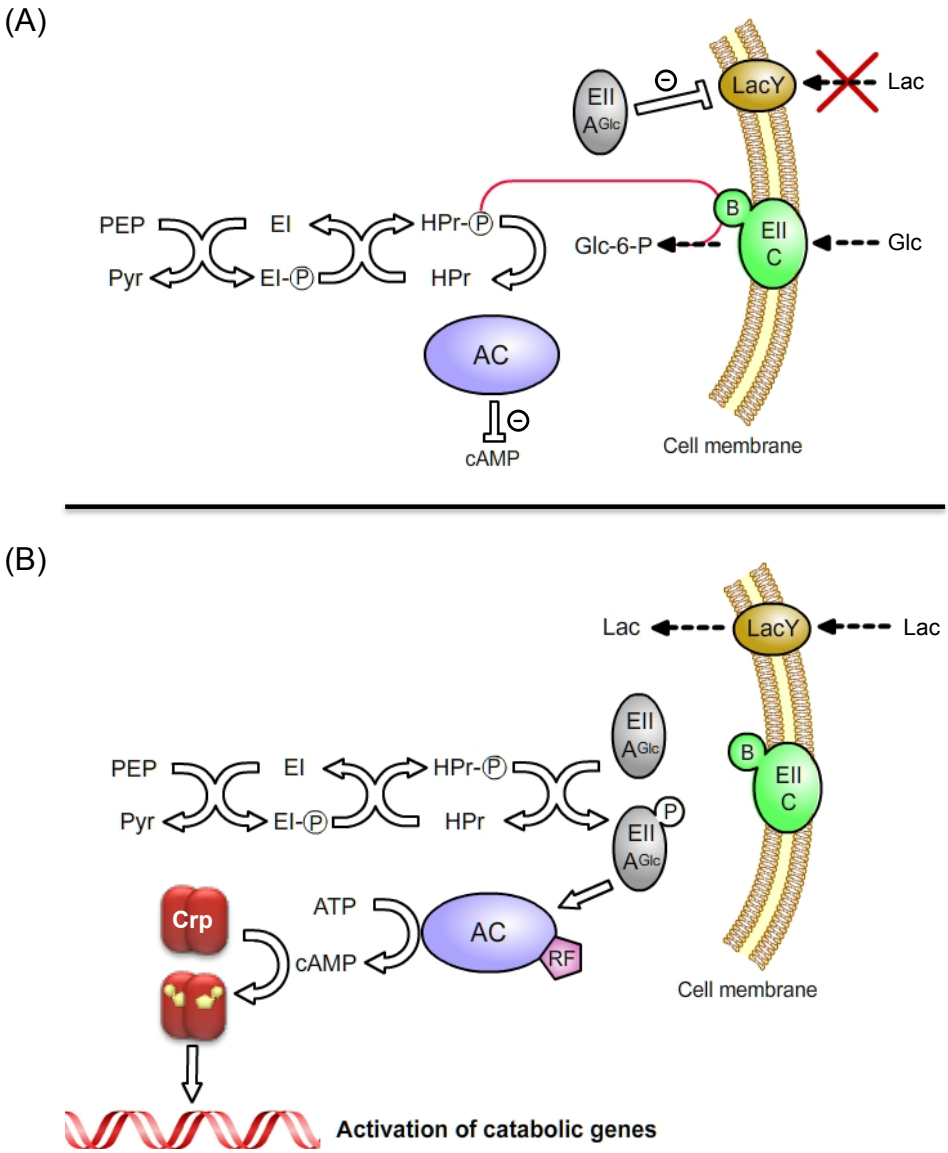


Fig. 3. Classical model of the Carbon Catabolite Repression (CCR) of carbohydrates in *E. coli*. **(A)** The glucose specific EIIA of the PTS-phosphotransferase system mediates the CCR mechanism in *E. coli.* When phosphorylated, $EIIA^{Glc}$ binds and activates the adenylate cyclase enzyme (AC) to produce cAMP. Activation of AC requires also the interaction of an unrecognized regulatory factor of the cell cytoplasm (*RF*). In the presence of a mixture of sugars, glucose (Glc) regulates the flow of the phosphate from PEP to the carbohydrate simultaneously with its transport by the EIIBC components. This prevents the $EIIA^{Glc}$ to become phosphorylated by the PTS. Nonphosphorylated $EIIA^{Glc}$ interacts with the transporters of non-preferred carbon sources such as lactose permease (LacY), impeding the uptake of alternative carbon sources by the mechanism known as *inducer exclusion*. **(B)** When glucose is consumed, the $EIIA^{Glc}$ becomes phosphorylated. Phospho-$EIIA^{Glc}$ activates the adenylate cyclase, increasing cellular cAMP concentrations. High concentrations of the cyclic nucleotide trigger the formation of cAMP-Crp complex, which binds and stimulates transcription of catabolic operons for consumption of alternative carbon sources.

Carbohydrate specificity resides in EII, and hence, bacteria usually contain many different EII complexes, consisting in one or two integral membrane domains (C and D) and two hydrophilic domains (A and B; Deutscher *et al.*, 2006). Regulation of CCR in *E. coli* is thus brought about by the IIA component of the glucose-specific PTS ($EIIA^{Glc}$, encoded by *ccr* gene). In the classical model of CCR in enterobacteria (Fig. 3), when cells are faced with a mixture of carbohydrates, the uptake of the preferentially consumed PTS substrate like glucose increases the level of dephosphorylated $EIIA^{Glc}$ (Reviewed in Deutscher *et al.*, 2006; Deutscher, 2008; Gorke & Stulke, 2008). This occurs because the phosphate group transferred from phosphoenolpyruvate (PEP) through PTS system is drained towards transport of the preferred carbon source (glucose-6-phosphate in Fig. 3A). Phosphorylated (but not unphosphorylated) $EIIA^{Glc}$ exerts its regulatory role by activating the cAMP-producing enzyme adenylate cyclase (AC; Kolb *et al.*, 1993). Recently, it was discovered that this activation is also dependent on an uncharacterized regulatory factor *(RF)* in the cell cytoplasm that interacts with the AC, with $EIIA^{Glc}$~P or with both proteins (Park *et al.*, 2006). Therefore, the presence of glucose lowers the intracellular concentrations of this cyclic nucleotide. Also, unphosphorylated $EIIA^{Glc}$ can bind to a number of enzymes involved in the transport and metabolism of non-PTS carbon sources (e.g. lactose, melibiose, maltose and glycerol), and thereby inhibits the utilization of these carbon sources, a process called inducer exclusion (Deutscher *et al.*, 2006; Deutscher, 2008; Gorke & Stulke, 2008). Once the preferred carbon source is exhausted, the adenylate cyclase is activated by $EIIA^{Glc}$~P and the regulatory factor *RF*. This increases the synthesis of cAMP, that binds to Crp and therefore, the cAMP-Crp complex activates the promoters of many catabolic genes and operons (Fig. 3B).

2.3 Transcription activation at simple Crp-dependent promoters

The cAMP-Crp complex binds at a variety of distances upstream of the transcription start site of several promoters to activate transcription of thereby encoded genes. This spacing has a central role in determining the mechanism of protein-protein interaction between the TF and the RNAP (Gaston *et al.*, 1990). Thus, transcription activation at simple Crp-dependent promoters are divided in three classes (Fig. 4; reviewed by Busby & Ebright, 1999).

[i] Transcription activation at class I Crp-dependent promoters require only one cAMP-Crp complex interacting with the specific DNA-binding site of Crp centered near positions -93, position -83, position -72 or position -62 from transcription start point (Fig. 4A). Location at these positions affords the DNA site for Crp and the DNA site for the RNA polymerase to be on the same face of the DNA-helix (Gaston *et al.*, 1990; Busby & Ebright, 1999). Transcription activation in this type of promoters involves a direct protein-protein interaction between Crp and the C-terminal domain of RNAP-α subunit (αCTD) through a determinant consisting of residues 156-164 of Crp and known as *activating region 1* (AR1; Zhou *et al.*, 1993; Busby & Ebright, 1999). AR1 interacts directly with residue 287 of αCTD and facilitates the binding of this RNAP subunit to DNA. Protein-DNA interaction is mediated by the residue 265 of αCTD. Finally, the residue 261 of αCTD (located on the face of αCTD opposite from Crp) is proposed to interact with the σ factor (Benoff *et al.*, 2002; Busby & Ebright, 1999). The interactions between Crp, RNAP and DNA, increase the affinity of RNAP for the promoter region, resulting in formation of the RNAP-promoter closed complex and, thus, in an increase of transcription initiation (Busby & Ebright, 1999).

[ii] At class II Crp-dependent promoters, one DNA site for Crp is centered near position -41.5 from transcription start point, thus overlapping the DNA site for RNAP (Fig. 4B; (Gaston *et al.*, 1990; Busby & Ebright, 1999). In this kind of promoters, Crp activates transcription through two different set of interactions: the first interaction involves the previously described *activating region* 1 (Bell *et al.*, 1990; Zhou *et al.*, 1994; Busby & Ebright, 1999). AR1 interacts with the 287 determinant of one of the two copies of αCTD, facilitating the binding of this subunit to the DNA segment upstream Crp (Fig. 4B; Busby & Ebright, 1999) The second interaction requires a class II-specific determinant consisting in residues His 19, His 21, Glu 96 and Lys 101 located in the N-terminal CBD of Crp (*activating region* 2, AR2; Niu *et al.*, 1996; Busby & Ebright, 1999). This AR2 interacts with the N-terminal domain of RNAP-α subunit (αNTD), specifically in the determinant formed by residues 162-165. Thus, interaction of Crp-AR1 with αCTD facilitates the formation of the RNAP-promoter DNA closed complex, while AR2 facilitates de transition of closed complex to open complex (Niu *et al.*, 1996; Busby & Ebright, 1999). Additionally, there is a third non-native activating region at class-II Crp dependent promoters triggered by the mutation of the inhibitory determinant composed of residue

Lys52 mainly by a neutral or negative charged amino acid (*activating region* 3, AR3; Busby & Ebright, 1999; Rhodius & Busby, 2000b). This inhibitory effect is proposed to occur mainly due to an unfavourable interaction of Lys52 side chain that clashes with RNAP.

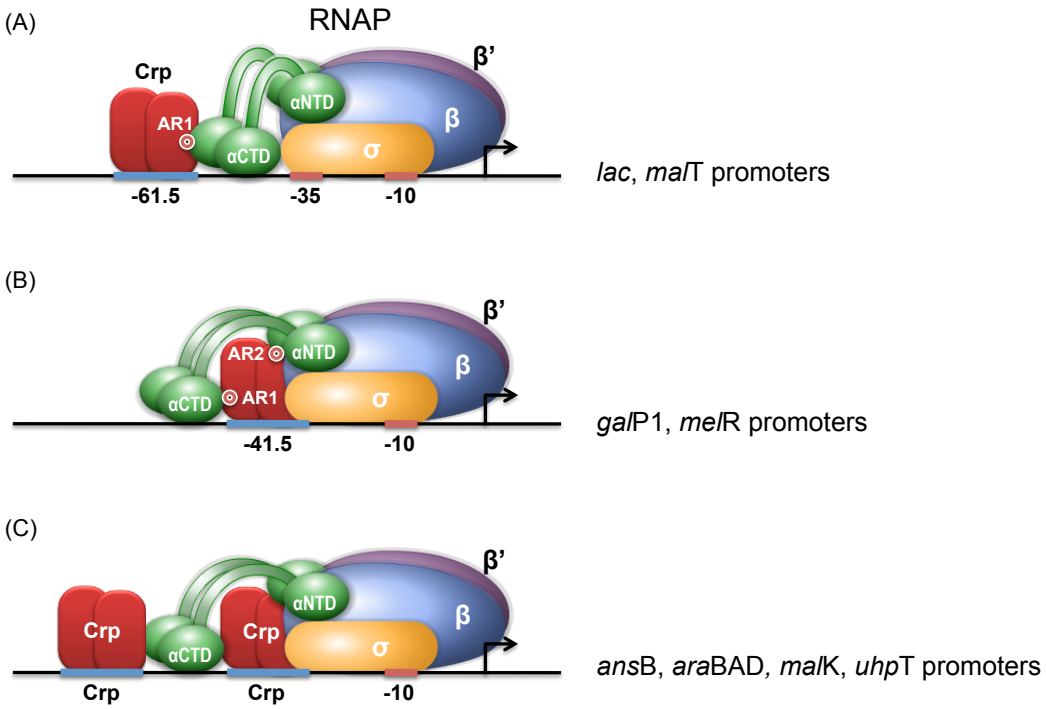


Fig. 4. Mechanisms of transcription activation at Crp-dependent promoters. **(A)** Ternary complex of Crp, RNA polymerase (RNAP) and the DNA of class I-dependent promoter having the DNA site for Crp centered at position -61.5. Transcriptional activation requires direct protein-protein interaction between *activating region* 1 of Crp (AR1) and the 287 determinant of one copy of RNAP-αCTD. The AR1–αCTD interaction facilitates the binding of αCTD to the DNA segment immediately downstream of Crp and to σ^{70} region 4 bound at -35 element. **(B)** Ternary complex of Crp, DNA and RNAP at class II Crp-dependent promoters. Transcription activation requires the location of the Crp-site centered near position -41.5. Protein-protein interaction between Crp-RNAP requires two activating regions at Crp. The AR1 of the upstream subunit of Crp interact with residue 287 of one copy of αCTD and facilitates the binding of αCTD to DNA. The *activating region* 2 (AR2) of the downstream subunit of Crp interacts with one copy of αNTD. **(C)** Quaternary complex of multiple Crp subunits, RNAP and a class III Crp-dependent promoter having an upstream Crp dimer operating by a class I mechanism, and the downstream Crp dimer functioning by a class II mechanism. For details see text. Adapted from Busby & Ebright (1999).

In the putative model of transcription activation at AR3, substitution of inhibitory Lys52 relieves this clash, allowing the interaction of the negatively charged residues D53, E54,

E55 and E58 with the positively charged amino acids K593, K597, R599 and R596 located at the C-terminal region of σ^{70} subunit of RNAP (Rhodius & Busby, 2000b; Rhodius & Busby, 2000a).

[iii] Finally, transcription activation at class III dependent promoters requires multiple activator molecules for full transcription activation (Fig. 4C). This synergistic transcription activation could be obtained by multiple Crp dimers placed at various class I positions (e.g. -103, -93 and/or -62 position from transcription star site). Similarly a Crp dimer located upstream could function by a class I mechanism, while another Crp dimer located downstream near -41.5 position functions by a class II mechanism (Busby & Ebright, 1999). Also, synergistic transcription activation could be obtained by interaction of Crp and other activators (Busby & Ebright, 1999). Amongst this kind of class III Crp-dependent promoters, a particular case is the coregulation exerted by Sxy and Crp over *ppd*A or *yrf*D genes. These promoters are regulated by Crp positioning at a non-canonical Crp site (called Crp-S sites) with the core half sequence 5'-TGCGANNN instead consensus 5'-TGTGANNN. Thus, binding of Crp to the non-canonical Crp-S site requires the interaction of the Sxy factor (Cameron & Redfield, 2006).

3 Adenylate cyclase (CyaA)

As stated above, cyclic nucleotides are found amongst the most important second messengers throughout the entire Tree of Life (Gomelsky, 2011). These signalling molecules are synthetized by nucleotide cyclase enzymes. While the adenylate cyclase (AC) catalyses the production of 3'-5'-cyclic adenosine monophosphate (cAMP) from ATP, the homologous guanylate cyclase (GC) catalyses the formation of 3'-5'-cyclic guanosine monophosphate (cGMP) from GTP (Danchin, 1993). At least, six classes of nucleotide cyclases have been described: The class I or enterobacterial cyclase is represented by the CyaA adenylate cyclase of *E. coli* (Danchin, 1993). These enzymes have only been found in γ- and δ-proteobacteria (Linder, 2008). Class II, or the Calmodulin activated toxic class of cyclases, includes a group of toxic adenylate cyclases of pathogens like *Bacillus anthracis*, *Bordetella pertussis* or *P. aeruginosa* (Danchin, 1993). The class III or the universal class of nucleotide cyclases is an ancestral group of purine cyclases that comprises adenylate and guanylate cyclases present in bacteria, archaea and eukaryotes (Danchin, 1993). The class

IV was initially identified in hyperthermophilic archaebacteria and is represented by the AC2 of *Aeromonas hydrophila* (Sismeiro *et al.*, 1998). Little is know about class V and class VI nucleotide cyclases, which are represented by the adenylate cyclases of the anaerobic *Prevotella runimicola* and *Rhizobium etli*, respectively (Shenroy & Visweswariah, 2004).

The CyaA of *E. coli* is probably the only class I AC that has been biochemically characterized to some extent (Linder, 2008). CyaA is a soluble monomeric protein of 848 amino acids and ~95 kDa of molecular weight (Yang & Epstein, 1983). The modular enzyme is composed of two functional domains, an amino-terminal catalytic domain and a carboxy-terminal regulatory domain (Roy *et al.*, 1983; Linder, 2008; Park *et al.*, 2006). The catalytic activity of the N-terminal domain is elicited within the first 420 amino acids of CyaA, where 12 residues were identified to be essential for enzymatic activity (Linder, 2008). The proposed mechanism for cAMP synthesis involves the binding of two metal ions (Mg^{2+}) to two aspartate residues (Asp 114 and Asp 116) within the catalytic domain (Roy *et al.*, 1983; Linder, 2008). These metal-cofactors catalyse the cyclization reaction. ATP is proposed to be discerned from ADP and AMP via Ser 103, which binds the γ phosphate and provides the substrate binding energy (Linder, 2008). On the other hand, the C-terminal regulatory domain is essential for metabolic activation of the adenylate cyclase by the phosphorylated-$EIIA^{Glc}$ and the *RF* factor of the cell cytoplasm (Park *et al.*, 2006).

4 cAMP phosphodiesterase (cAMP PDE)

The intracellular concentration of cAMP is determined by its synthesis through adenylyl cyclases (see above), degradation, and the efflux across the cell membrane. Conversely to the function of adenylate cyclase, the turnover of intracellular cAMP is carried through the specialized enzyme cyclic 3',5'-AMP phosphodiesterase. The cyclic nucleotide phosphodiesterases (PDE) catalyzes the hydrolytic cleavage of the 3′-phosphodiester bond of the cyclic nucleotide, resulting in formation of the corresponding inactive 5′-monophosphate (Essayan, 2001; Richter, 2002). Like adenylate cyclases, cyclic nucleotide phosphodiesterases are found in all branches of life and encompass a family of enzymes that are grouped into three classes based on their primary amino acid sequence: Class I include all mammalian PDE described as well as several genes identified in *Drosophila,*

Caenorhabditis, Dictyostelium discoideum and yeast. The phosphodiesterase class II is comprised of a few enzymes from *Saccharomyces cerevisae, Dictyostelium discoideum, Schizosaccharomyzes pombe, Candida albicans,* and a periplasmic PDE from *Vibrio fischeri.* Finally, the newly identified class III PDE includes most of the phosphodiesterases found in bacteria (Richter, 2002). This comprises the phosphodiesterases of *E. coli* (Imamura *et al.*, 1996; Richter, 2002), *Haemophilus influenzae* (Macfadyen *et al.*, 1998) , *Mycobacterium* tuberculosis (Shenoy *et al.*, 2007), *Anabaena* sp. PCC 7120 (Fujisawa & Ohmori, 2005) and *Pseudomonas aeruginosa* (Fuchs *et al.*, 2010b). Richter (2002) found that, despite high similarity between class III phosphodiesterases, only 13 amino acid residues could be identified as absolutely conserved in this class of enzymes (detail of this residues can be found in Fig. 6). These residues appear to be organized in five sequence clusters that correspond very well to a sequence signature of the formula D-(X)n-GD-(X)n-GNH[E/D]-(X)n-H-(X)n-GHXH (where X is any residue, and *n* is the number of repeats). This is known from purple acid phosphatases, which catalyse the hydrolysis of activated phosphoric acid esters and anhydrides. They contain a binuclear Fe^{3+}-Me^{2+} catalytic center in their active site (where Me^{2+} can be occupied by Fe, Zn, or Mn). Imamura *et al.* (1996) identified that the addition of iron ions stimulated the activity of CpdA phosphodiesterase of *E. coli*. Furthermore, X-ray crystallography of *M. tuberculosis* dimeric phosphodiesterase revealed that this protein contain a Fe^{3+}-Mn^{2+} coordinated in the core active site (Shenoy *et al.*, 2007). Thus, although not fully understood, the mechanism of action of class III PDE could be similar to the catalytic activity of purple acid phosphodiesterases (Richter, 2002; Fuchs *et al.*, 2010b).

5 Virulence factor regulator (Vfr) of *Pseudomonas aeruginosa*

In view of the massive involvement of Crp in the control of metabolic functions in *E. coli* (see above), it has been generally assumed that *bona fide* orthologues found in many eubacterial genomes could fulfil the same role. This notion changed with the discovery in 1994 of the so-called virulence factor regulator (Vfr), a protein of the opportunistic pathogen *Pseudomonas aeruginosa* that is virtually identical to Crp (67% identity and 91% similarity) but lacks functional similarity in respect to the metabolism of this bacterium (West *et al.*, 1994; Suh *et al.*, 2002). Moreover, whole transcriptome and proteome analysis

of *vfr* mutants demonstrate that Vfr is a global transcriptional regulator that controls the expression of more than 200 genes and at least 60 proteins (Wolfgang *et al.*, 2003; Suh *et al.*, 2002). Virtually none of the large list of genes controlled by Vfr is related with metabolic traits in this bacterium (Wolfgang *et al.*, 2003). Instead, Vfr exerts a global regulatory effect on the production of multiple surface-exposed and secreted virulence factors. These include the production of exotoxin A (ETA or ToxA), type IV pili (Tfp), flagellar biosynthesis, type III secretion system (T3SS), and the *las* quorum sensing system (including *las*R and *las*B) which, in turn, controls the expression of hundreds of additional genes, including multiple virulence factors (West *et al.*, 1994; Albus *et al.*, 1997; Dasgupta *et al.*, 2002; Wolfgang *et al.*, 2003; Kanack *et al.*, 2006). Vfr also exerts a positive autoregulation over its own expression and over the promoter of the *cpd*A gene, encoding a cyclic 3',5'-AMP phosphodiesterase (Fuchs *et al.*, 2010a; Fuchs *et al.*, 2010b). This provides a feedback mechanism for controlling cAMP levels and a fine-tuning virulence factor expression.

Vfr acts mainly in concert with cAMP as effector molecule. However, there is some evidence that Vfr also can operate though cAMP-independent mechanisms, since cAMP is not required for Vfr binding to the *las*R promoter *in vitro* or for activation of the *las*R promoter activity *in vivo* (Fuchs *et al.*, 2010b). In *P. aeruginosa*, two different classes of adenylate cyclases produce cAMP (Wolfgang *et al.*, 2003). Most of the cyclic nucleotide is produced via the membrane-bound CyaB, a class II adenylate cyclase. The rest of cAMP contribution is produced by the cytoplasmic class I CyaA (Danchin, 1993; Wolfgang *et al.*, 2003). Both ACs respond to environmental signals as calcium concentration and host cell contact. Thus, the cAMP-signalling pathway in *P. aeruginosa* seems to be responding to completely different signals in comparison with the metabolic regulation of cAMP observed in *E. coli* (Wolfgang *et al.*, 2003).

6 cAMP-Crp system in *Pseudomonas putida*

Bacteria from the genus *Pseudomonas* were initially described in 1894 by W. Migula as Gram-negative, polarly flagellated strictly aerobic rods. Defined in this way, the genus was very heterogeneous and did not allow a clear distinction from other Gram-negative bacterial groups (Palleroni, 1984; Palleroni, 2008). Therefore, a more precise criteria for

classification was defined first with the inclusion of the morphological and phenotypical characteristics of the bacterium and later with the implementation of nucleic acid studies such as DNA-rRNA hybridization, rRNA oligonucleotide cataloguing, 5S rRNA sequencing and finally by 16S rRNA sequencing (Stanier *et al.*, 1966; Moore *et al.*, 1996; Palleroni, 2008). This later method, led to the clustering of *Pseudomonas* within the γ subclass of Proteobacteria, now recognized as the class Gammaproteobacteria (Palleroni & Moore, 2004). The genus *Pseudomonas* collectively exhibit a highly diverse range of activities: they are extremely versatile metabolically, physiologically and genetically and engage in many critically important environmental activities, such as element cycling, degradation and recycling of biogenic and xenobiotic organic compounds, food spoilage, growth promotion and protection from pathogens of plants (Timmis, 2002). They also include important human opportunistic pathogens as *P. aeruginosa* or plant pathogens as *P. syringae* (Klockgether *et al.*, 2008; Rico *et al.*, 2011).

Pseudomonas putida is a non-pathogenic bacterium frequently isolated from most temperate soils (particularly polluted soils), waters or plant rhizosphere. It is a nutritional opportunist *par excellence* and a paradigm of metabolically versatile microorganisms that recycle organic wastes in aerobic compartments of the environment (Timmis, 2002; Martins dos Santos *et al.*, 2004). *P. putida* strain KT2440 is probably the best characterized saprophytic Pseudomonad that has retained its ability to survive and function in the environment (Nelson *et al.*, 2002; Martins dos Santos *et al.*, 2004). This bacterium is a derivative of *P. putida* mt-2 (originally isolated in Japan by K. Hosokawa as *P. arvilla* mt-2) that has lost the TOL plasmid (Nakazawa, 2002), which encodes genetic pathways for degradation of toluene, *m*- and *p*-xylene in two catabolic operons (Assinder & Williams, 1990; Ramos *et al.*, 1997). The wide metabolic versatility of this bacterium, joint with the capacity to degrade xenobiotic compounds, its robustness, rapid growth, the availability of genome sequence, ease of handling in the laboratory and its amenability to genetic analysis and manipulation, have resulted in *P. putida* becoming a laboratory *workhorse* for research on soil bacteria and for biotechnological applications such as bioremediation and biocatalysis (Jimenez *et al.*, 2002; Timmis, 2002; Nelson *et al.*, 2002; Wackett, 2003).

Although *E. coli* Crp was the first transcriptional activator ever described, the information in the literature on the homologous protein of *P. putida* is scarce. Milanesio and colleagues

(2011) reported the presence in the genome of *P. putida* KT2440 of singular orthologues of *cya*A and *crp* genes of *E. coli*, encoding a genuine Adenylate cyclase and cAMP receptor protein, respectively. Despite the high degree of identity between $Crp_{P.\ putida}$ and $CyaA_{P.\ putida}$ with the similar proteins of *E. coli*, deletion of *crp* or *cya*A in the soil bacterium lead to virtually unnoticeable changes in the gross metabolic conditions of the cell, as consumption of a battery of carbon and nitrogen sources remained unaltered with respect to the wild-type *P. putida*. Moreover, deletion of both genes leads the mutants to acquire a higher tolerance to pyrophosphate and protamine as well as a noticeable resistance to colistin. At the same time, they lost concurrently the ability to use a large number of dipeptides as the only N source and became more sensitive to some antibiotics and antimetabolites (Milanesio *et al.*, 2011). Besides the regulatory role in the resistance to antibiotics and toxic compounds, *crp* and *cya*A mutations seems to affects other cell surface-related functions as cell motility and biofilm formation (Milanesio, 2007). Similarly, Daniels *et al.* (2010) showed that deletion of *crp* in the solvent-tolerant strain *P. putida* DOT-T1E did not alter markedly the growth of the bacterium in 35 different carbon sources, except when cells were grown with L-valine, L-tyrosine, decanoate, propionate and 2-phenylethylamine as the only C source. Additionally, they demonstrate that the growth of the *crp* mutant was impaired in cells growing with L-arginine, L-serine and the dipeptides Ala-Thr, Gly-Gln, Gly-Gly, Gly-Ser as the only nitrogen source. Finally, Herrera *et al.* (2012) showed some evidences that Crp could be implicated in the co-activation of the *phh*AB operon (involved in the metabolism of phenylalanine and tyrosine) together with the regulator PhhR and IHF.

7 The issue at stake

Fully understanding of the gene regulatory network is essential not only to know how bacteria thrive to changing environments in nature, but also to manipulate the gene expression in order to take advantage of these organisms for biotechnological purposes. In this regard, the metabolic versatility, easy handling in the laboratory, growing availability of genetic tools and robustness of *Pseudomonas putida,* make this organism a powerful agent that can be exploited for applications as bioremediation, biocatalysis or as a cell factory. Despite the significant role played by Crp proteins in Gram-negative bacteria since its

discovery more than 40 years ago, the regulatory duties of cAMP-Crp system in *P. putida* are not understood. Some evidences point out that cAMP-Crp regulation is related with cell envelope functions and with the consumption of amino acids as carbon and nitrogen source. Notably, it seems that in *P. putida* this system does not have any relation with the generic regulation of carbon and energy metabolism with which this system has been linked in enterobacterial organisms. In order to clarify this picture, we have investigated the components of the cAMP-Crp system of gene regulation of *P. putida* from a phenotypic, biochemical and genetic point of view. To this end, in this work we have addressed the objectives spelled out next.

II. Objectives

Objectives

General objective:

To investigate the regulatory mechanisms of the cAMP-Crp system of gene expression in *Pseudomonas putida*, as well as the functions that are subject to it.

Specific objectives:

1. To characterize genetically and phenotypically the *crp, cya*A and *cpd*A genes of *P. putida*, as well as the protein products of each one of these genes.
2. To survey the intracellular levels of the second metabolite cAMP in *P. putida.*
3. To purify and characterize biochemically the $Crp_{P.\ putida}$ and its modulation by cAMP.
4. To identify the genes direct or indirectly regulated by the cAMP-Crp system in *P. putida.*

III. Materials and Methods

1 Culture conditions and media

Unless otherwise indicated, the *E. coli* and *P. putida* cells were respectively grown at 30°C and 37°C in either rich Luria-Bertani (LB) medium or in synthetic mineral M9 medium (Sambrook *et al.*, 1989) supplemented with 0.2% of the C-sources indicated in each case and with 170 rpm orbital shaking. Additionally, 0.2% (w/v) glucose (Sigma-Aldrich) was supplemented to LB for cells grown for MBP-Crp protein purification, according to the pMAL™ Protein Fusion and Purification System (New England Biolabs) as described below. Likewise, 0.0005% of thiamine was added in the specific minimal medium for growing the *E. coli* strains. For solid media preparation, LB and M9 medium were supplemented with 1.5% (w/v) of Bacto Agar (Pronadisa). For *crp* and *cya*A complementation experiments, indicator plates were prepared by adding 1.0% (w/v) of maltose to MacConkey agar base (Miller, 1972). The growth of the bacterial strains on M9 minimal medium was measured in microtiter plates by turbidometry at 600 nm using the Victor 2 multireader spectrophotometer (Perkin Elmer). Where required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the media as inducer of P*lac*-derived promoters. The following antibiotics were added to the cultures to retain plasmids and to select for plasmid cointegration, as indicated in each case: ampicillin (Ap) 150 μg/ml, chloramphenicol (Cm) 40 μg/ml, kanamycin (Km) 50 μg/ml and tetracycline (Tc) 8 μg/ml.

2 Bacterial strains

The bacteria used in this work are described in Table 1 with their corresponding genotype. All *P. putida* strains generated were derived from the reference strain KT2440 (Nelson *et al.*, 2002). The *E. coli* strains CC118, DH5α and JM109 were used for routine maintenance of plasmids. Similarly, the replication of plasmids with the oriR6K suicide origin of replication (see Table 2) was done in DH5α*λpir* and CC118*λpir* phage lysogen strains of *E. coli*. For *crp* and *cya*A complementation assays, the *E. coli* strain of reference was W3110 on which various mutations were engineered, as will be described.

Table 1. Description of the Bacterial strains used in this study

Strain	Description	Reference
***Pseudomonas putida* strains**		
KT2440	Prototrophic, wild-type strain derived of *P. putida* mt-2 without pWWO plasmid	Nelson *et al.*, 2002
KT2440 Δ*crp*	KT2440 derivative with a full deletion of *crp*	This work
KT2440 Δ*cya*A	KT2440 derivative with a full deletion of *cya*A	This work
KT2440 *cpd*A	Km^R, Rif^R. KT2440 derivative carrying a chromosomal insertion of the mini-Tn5 Km in PP_4917 (*cpd*A)	Duque *et al.*, 2007
MAD-1	Tel^R, Rif^R KT2442 with a chromosomal insertion of mini-*Tn*5 carrying a *xylR*, *Pu-lacZ* fusion	Fernandez *et al.*, 1995
MAD-1 *crp*	MAD1 derivative, *crp::xylE*	Milanesio *et al.*, 2011
MAD-1 *cya*A	MAD1 derivative, *cya*A*::km*	Milanesio *et al.*, 2011
***Escherichia coli* strains**		
JM109	F´, *traD36, proA*$^+$*B*$^+$, *lacI*^{q}, Δ(*lacZ*)*M15*/ Δ(*lac-proAB*), *glnV44 e14*$^-$, *gyrA96, recA1, relA1, endA1, thi, hsdR17*	Yanisch-Perron *et al.*, 1985
CC118	F$^-$, Δ(*ara-leu*)7697, *araD*139, Δ(*lac*)X74, *phoA*Δ20, *galE, galK, thi, rpsE, rpoB, argE*(Am), *recA1*	Manoil & Beckwith, 1985
CC118λ*pir*	λ*pir* phage lysogen of CC118	de Lorenzo & Timmis, 1994
DH5α	F$^-$, *supE44, ΔlacU169, (ϕ80 lacZDM15), hsdR17, (rk*$^-$ *mk*$^+$*), recA1, endA1, thi1, gyrA, relA*	Hanahan & Meselson, 1983
HB101	Sm^r, *hsdR*$^-$*M*$^+$, *pro, leu, thi, recA*	Sambrook *et al.*, 1989
DH5αλ*pir*	λ*pir* phage lysogen of DH5α	Lab collection
W3110	Prototrophic, F$^-$, l$^-$, IN (*rrnD-rrnE*)1, *rph-1*	Hayashi *et al.*, 2006

W3110 *cyaA*	*cyaA* deletion derivative of W3110 strain	This work
W3110 *crp*	*crp* deletion derivative of W3110 strain	This work
W3110 *crp cyaA*	Double *crp cyaA* deletion derivative of W3110	This work
TP610A	F^-, *thi-1*, *thr-1*, *leuB6*, *pro*, *lacY1*, *tonA2l*, *supE44*, λ-*hsdR hsdM recBC lop-11 lig*$^+$ *cya-610,* spontaneous mutant of strain TP610 with hypersensitivity to low levels of cAMP	Crasnier & Danchin, 1990

3 Plasmids

A brief description of the plasmids employed in this work is listed in Table 2. The suicide vector pEMG and its derivatives with R6K origin of replication were used to construct full deletions of *crp* and *cya*A genes both in *E. coli* and *P. putida* (Martinez-Garcia & de Lorenzo, 2011). The plasmid pRK600 was used in bacterial conjugations. The remaining plasmids were used as expression vectors.

Table 2. Plasmids employed in this work

Plasmid	Description	Reference
pRK600	Cm^R; *ori*V ColE1, *tra*$^+$*mob*$^+$ of RK2, helper plasmid for mobilization in tripartite conjugations	Kessler *et al.*, 1992
pEMG	Km^R, *ori*R6K, suicide plasmid with two I-SceI sites flanking the *lac*Zα polylinker	Martinez-Garcia & de Lorenzo, 2011
pSW-1	Ap^R, *ori*RK2, *xylS*, bearing a *Pm→I-sce*I transcriptional fusion	Wong & Mekalanos, 2000
pARCrp8	Km^R, *ori*R6K, suicide pEMG carrying the upstream and downstream (TS1-TS2) flanking regions of *E. coli crp* gene	This work
pARCrp9	Km^R, *ori*R6K, suicide pEMG carrying the upstream and downstream (TS1-TS2) flanking regions of *P. putida crp* gene	This work

pJΔcyaA	Km^R, *ori*R6K, suicide pEMG carrying the upstream and downstream (TS1-TS2) flanking regions of *P. putida cya*A gene	This work
pACBSR	Cm^R, I-*Sce*I and λ*red* recombinase expression plasmid with arabinose-inducible expression	Herring *et al.*, 2003
pUC18Not	Ap^R, *oriV* ColE1, cloning vector, polylinker flanked by *Not*I sites	Herrero *et al.*, 1990
pVLT31	Tc^R, *oriV* RSF1010, broad-host range LacIq/IPTG inducible expression vector	de Lorenzo *et al.*, 1993
pGEM-T	Ap^R, multicopy cloning vector	Promega
pVTR-A	Cm^R, *oriV* PSC101, LacIq/IPTG inducible expression cassette inserted as a *Not*I segment	Perez-Martin & de Lorenzo, 1996
pCA24N	Cm^R, *oriV* ColE1 cloning and IPTG-inducible expression vector	Mori *et al.*, 2000
pCA24N *crp*	Cm^R, pCA24N inserted with *crp* gene of *E. coli*	Mori *et al.*, 2000
pMZC5	Tc^R, pVLT31 inserted with the *P. putida cya*A gene sequence	Milanesio *et al.*, 2011
pMZC6	Ap^R, pUC18Not inserted in the *Eco*RI/*Hind*III sites with the *P. putida cyaA* gene cassette of pMZC5	Milanesio *et al.*, 2011
pARV1	Ap^R, pUC18Not inserted with the *P. putida crp* gene sequence. The encoded Crp is added with terminal 6xHis.	This work
pARV2	Tc^R, pVLT31 bearing with the *P. putida crp* gene sequence assembled in pARV1	This work
pARCrp10	Tc^R, pVLT31 cloned with the *Eco*RI/*Pst*I fragment of pCA24Ncrp that encodes the *crp* gene of *E. coli* bearing the sequence of an N-terminal 6xHis	This work
pARC2	Cm^R. pVTR-A with its *Not*I insert altogether replaced by a segment bearing the *cya*A gene of *E. coli* expressed through its native transcription and translation signals.	This work

pDIA5240	Ap^R, *oriV* ColE1, expression plasmid for the catalytic subunit of the adenylate cyclase gene of *Bordetella pertussis*	Ladant *et al.*, 1992
pARC3	Tc^R, pVLT31 inserted with the *B. pertussis cya*A gene amplified from plasmid pDIA5240	This work
pARCrp4	Ap^R, pGEM-T cloned with PCR fragment of *P. putida crp* gene	This work
pMAL-C2T	Ap^R, commercial pMAL-c2X (MBP gene fusion vector) with Factor Xa cleavage site modified for the thrombin protease recognition motif	Arce-Rodriguez *et al.*, 2012
pARCrp5	Ap^R, pMAL-C2T harbouring the MBP-Crp fusion protein	This work
pJCD01	Ap^R, *ori*ColE1, polylinker of pUC19 flanked by *rpo*C and *rrnB*T1T2 terminators	Marschall *et al.*, 1998
pJCD-Plac	Ap^R, pJCD01 cloned with the *lac* promoter of *E. coli*	This work
pSEVA225	Km^R, *ori*RK2, promoterless *lac*Z, vector for constructing transcriptional fusions	Lab collection
pSEVA225T	Km^R, *ori*RK2, promoterless *lac*Z, vector for cloning *in-frame* translational fusions with *lac*Z	Lab collection
pARPcrp1	Km^R, *ori*RK2, *lac*Z translational fusion to the *crp* promoter region plus 30 codons from its ATG	This work
pARPcyaA1	Km^R, *ori*RK2, *lac*Z translational fusion to the *cya*A promoter region plus 30 codons from its ATG	This work
pARPcyaA2	Km^R, *ori*RK2, transcriptional fusion of the *crp* promoter region to *lac*Z	This work

4 Recombinant DNA techniques

General methods for DNA manipulation were performed with standard protocols described previously (Sambrook *et al.*, 1989). Specific chromosomal segments of *P. putida* and *E. coli* were amplified from genomic DNA by means of polymerase chain reactions (PCR) using 100 ng of genomic DNA or 10 ng of plasmid as templates in a buffer

containing 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates (dNTP), 0.2 μM of oligonucleotides (indicated in each case) and 1 U of DNA polymerase. A list of the oligonucleotides used in this work is described in Table 3. PCR reactions were run by first setting an initial denaturalization 5 min at 94°C followed by 30 cycles of denaturalization (1 min, 94°C), annealing (1 min, 58°-64°C), extension (1-3 min at 72°C) and final extension (10 min, 72°C). Moreover, when PCR was carried out with high fidelity *Pfu* DNA Polymerase (Promega. Cat. Nº M774A), the extension temperature of reactions was increased to 75°C. In some cases, the same PCR reaction was directly run on a small amount of bacterial biomass picked from isolated colonies grown on agar plates. Where necessary, amplified DNA segments were purified from PCR or agarose gels using the QIAEX®II Gel extraction kit of QIAGEN (Cat. Nº 20021).

Plasmid DNA was extracted from bacterial cells by means of the alkaline lysis protocol using the commercial Wizard® *Plus* SV Minipreps DNA Purification kit (Promega. Cat. Nº A1460). The restriction endonucleases for DNA digestion and cloning were purchased from New England Biolabs, and used according to the manufacturer indications. Digested DNA was ligated by overnight incubation with T4 DNA ligase (Roche. Cat Nº 10481220001) at 16°C. All the DNA fragments amplified by PCR and cloned in each of the plasmids listed in Table 2 were verified by sequencing.

The mobilization of plasmidic DNA into *E. coli* cells was carried out by preparation of competent cells using the $CaCl_2$ method (Sambrook *et al.*, 1989), or by electroporation as described by Wirth *et al.* (1989). In the case of *P. putida*, plasmids were incorporated by conjugative triparental mating using the *E. coli* HB101 (pRK600) as helper strain (de Lorenzo & Timmis, 1994) or by electroporation of cells previously washed and concentrated with 300 mM sucrose as in Choi *et al.* (2006).

Table 3. Oligonucleotides designed in this Thesis for PCR amplification. The restriction site sequence (in parenthesis) is underlined in each case.

Name (restriction site)	Sequence (5'-3')
aaPpucrpUp-F (*Eco*RI)	TTAGGAATTCGCGCTTCTTTCAGCCCACGC
aaPpucrpUp-R	GCCGAGTTGCCCAAGGACTAGCACGAAATCCTGTTCGATATGAC
aaPpucrpDown-F	CTAGTCCTTGGGCAACTCGGC
aaPpucrpDown-R (*Sal*I)	ATACTGTCGACTGCTGCTGCGAGAAACCGGC
aaECcrpUP-F (*Eco*RI)	TTAGGAATTCCTTCACAATCGACCACATCCTGAC
aaECcrpUP-R	AACGCGCCACTCCGACGGGAGCGCGGTTATCCTCTGTTATAAGC
aaECcrpDown-F	TCCCGTCGGAGTGGCGCGTTAC
aaECcrpDown-R (*Sal*I)	TTAAGTCGACAACCAGTTAAACAATCCGTACCAGAG
aaECcyaA-F (*Not*I)	AAGGAAAAAAGCGGCCGCCAGTTCAACGACCAGGCCCG
aaECcyaA-R (*Not*I)	TAAGGAATTTGCGGCCGCGAGCCGCTGCACCAGGTATG
aaBPcyaA-F (*Eco*RI)	TTACGAATTCTCACACAGGAAACAGCTATGACC
aaBPcyaA-R (*Hind*III)	TGAAAAGCTTCTAGCGTTCCACTGCGCCCAG
vfrMyc1 (*Eco*RI)	CCGGAATTCCGGATTCAAGGAGATATATCCATGGTTGCCTCCGCC CTACCCGC
vfrHis-C (*Hind*III)	CCCAAGCTTGGGCTAGTGATGGTGATGATGATGGCGGGTACCGT GGAC
aaMBPVfr-F (*Eco*RI)	TGTTGAATTCATGGTTGCCTCCGCCCTACC
aaMBPVfr-R (*Hind*III)	TTATAAGCTTCTAGCGGGTACCGTGGACC
aaPlac-F	AGTCAAAATGCAAGTACTAGCTGATACCGCTCGCCGCAG
aaPlac-R	CTTGCGGCCGCCCAGCTTGGATATC
5'-PlacIVT (*Sca*I)	AAAAGTACTTGATACCGCTCGCCGCAG
3'-PlacIVT	ATCCAAGCTGGGCGGCCG
aaPcrp-F (*Eco*RI)	CGAATTCGCAGCTTTCCACTGCCTGGCGCGAC
aaPcrpTrad-R (*Bam*HI)	CGGGATCCATATTGCTTTTGGCGGTGTAGCGGC
aaPcyaA-F (*Eco*RI)	CGAATTCCAGCGGCACGTCACTGCAGATCACC
aaPcyaATrad-R (*Bam*HI)	CGGGATCCTTCAGATGCAGGAAGCGCGCACGC

aaPcyaA-R (*Bam*HI)	CGGGATCCTGCGCGCTCGGGCAGGCCATTGAAG
aaPpuCyaAUp1-F (*Eco*RI)	CGAATTCAACGCCTGTTTCTCACGTTC
aaPpuCyaAUp1-R	GCAGTCTTGTGCTGCGAGTTCAGAAGCTGTGGCGAGTTTAGC
aaPpuCyaATS2-F2	TGAACTCGCAGCACAAGACTGC
aaPpuCyaATS2-R (*Bam*HI)	CGGGATCCCAGGAGTCTGACTGGATCATGAGC

5 Protein techniques

Protein extracts were analysed by denaturing-polyacrylamide gel electrophoresis (SDS-PAGE) with 12-15% acrylamide/bisacrylamide (29:1) gel cast in a Mini-PROTEAN 3 Cell system (Bio-Rad), following standard protocols (Sambrook *et al.*, 1989). Whole cells and proteins were resuspended in a denaturing buffer containing 60 mM Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) glycerol, 1% (v/v) β-mercaptoethanol and 0.005% (w/v) bromophenol blue, and boiled for 5 min prior to loading. Gels were stained with a 0.05% (w/v) solution of Coomassie R-250 blue in methanol 50% (v/v) and acetic acid 10% (v/v), and de-stained in 10% (v/v) methanol with 7% (v/v) acetic acid. For immunodetection of Crp, protein samples were separated by electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF, inmobilon-P, Milipore) using a Trans-Blot® SD Semi-Dry transfer apparatus (Bio-Rad). Blotted membranes were blocked for 1 h at 22 °C (or 16 h 4°C) with 3% (w/v) skimmed milk, 1% (w/v) BSA, in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) containing 1% (v/v) Tween-20. The blots were subsequently incubated at room temperature in the same buffer (without milk) with anti-CRP rabbit serum (kindly provided by H. Aiba) diluted 1:1000-1:5000 for 1 h. After washing with PBS-Tween buffer, the membrane was incubated with an anti-rabbit antibody conjugated with peroxidase, washed again and then soaked in a buffer 100 mM Tris-HCl pH 8.0 with 1.20 mM luminol (Sigma), 42 mM luciferin (Roche) and 0.0075% H_2O_2 (v/v). After a 1 min in the dark, the blots were exposed to an X-ray film (X-OMAT, Kodak).

6 Construction of Δ*crp* and Δ*cya*A strains of *P. putida*

The deletion of *crp* and *cya*A genes in *P. putida* was obtained by the seamless genomic deletion system developed by Martínez-García and de Lorenzo (2011). In order to do so, the upstream (TS1, ~0.5 kb) and downstream (TS2, ~0.5 kb) flanking regions of *P. putida* *crp* and *cya*A genes were amplified respectively by PCR with primer pairs aaPpucrpUp-F/aaPpucrpUp-R and aaPpucrpDown-F/aaPpucrpDown-R (in the case of *crp*), and with primer pairs aaPpuCyaAUp1-F/aaPpuCyaAUp1-R and aaPpuCyaATS2-F2/aaPpuCyaATS2-R (in the case of *cya*A). The TS1 and TS2 products flanking each gene were then joined together by overlap extension (SOE)-PCR. The resulting ~1 kb TS1-TS2 fragment was *Eco*RI/*Sal*I digested and ligated within the same sites of pEMG, generating plasmids pARCrp9 for further deletion of *crp,* and pJΔcyaA for deletion of *cya*A. The plasmids were mobilized by conjugation to *P. putida* KT2440 previously electroporated with pSW-1 (the latter plasmid codifies the expression of I-SceI endonuclease under the control of Pm promoter). The cointegration of pARCrp9 and pJΔcyaA into the KT2440 chromosome was checked by Km and Ap (500 μg/ml) resistance, and by PCR amplification of the TS1-TS2 fragment (~1 kb). The cointegrated clones were grown overnight in 2 ml of LB medium with Ap (500 μg/ml) and 15 mM 3-methylbenzoate to drive the expression of I-SceI. Induced cells were plated in LB-Agar and the elimination of cointegrated plasmids was confirmed by streak replicates of single colonies in LB and LB-Km plates. The Km^S clones were selected and analysed by PCR of the TS1-TS2 fragment (~1 kb) to identify the individual deletion of *crp* and *cya*A genes. Finally, pSW-1 was diluted by three consecutive subcultures of the cells in liquid LB. The loss of this plasmid was confirmed by sensitivity to ampicillin in LB-Ap (500 μg/ml) plates.

7 *crp* and *cya*A complementation assays

7.1 Construction of single Δ*crp* and double Δ*crp* Δ*cya*A strains of *E. coli*

For construction of a reliable recipient of adenylate cyclases of various origins, we employed a *cya*A mutant of *E. coli* K12 strain W3110 in which the AC gene was cleanly deleted from the genome with the λ*red* recombinase method (Milanesio *et al.*, 2011). By the same token, the *crp* counterpart of W3110 strain was removed using the scarless deletion system

described previously (Martinez-Garcia & de Lorenzo, 2011) with several modifications outlined below. The deletion was done initially by PCR amplifying of the ~500 bp upstream (TS1) and downstream (TS2) flanking regions of $crp_{E.\ coli}$ with primer pairs aaECcrpUP-F/ aaECcrpUP-R and aaECcrpDown-F/aaECcrpDown-R, respectively. The resulting products (TS1 and TS2) were then joined together in a single DNA fragment by overlap extension (SOE)-PCR. The resulting ~1 kb TS1-TS2 segment was purified, digested with enzymes *Eco*RI/*Sal*I and ligated into the same sites of pEMG to generate the plasmid pARCrp8. This vector was isolated and mobilized by electroporation into *E. coli* W3110 cells, where pARCrp8 cointegration was selected by Km resistance and by PCR of TS1-TS2 segment with primers aaECcrpUP-F/ aaECcrpDown-R.

A group of pARCrp8-cointegrated colonies (Km^R) was pooled to prepare competent cells. These were transformed with pACBSR, which drives the expression of I-SceI under the control of an arabinose-dependent promoter (Herring *et al.*, 2003). The Km^R and Cm^R colonies were grown in 1ml of LB with 0.2% arabinose for one hour, whereupon the chloramphenicol was added to a final concentration of 40 μg/ml. The culture was grown another 7 hours and plated in LB-Agar. Replicates from individual colonies were patched in LB and LB-Km plates, to ascertain the loss of cointegrated plasmid. The Km^S clones were analysed by colony PCR as described previously to check the deletion of entire $crp_{E.coli}$ gene. Finally, the pACBSR was eliminated by three consecutive subcultures in liquid LB and the loss of the plasmid was confirmed by sensitivity to chloramphenicol in LB-Cm plates. This procedure was also applied to the W3110 Δ*cya*A strain for generating a double deletion host Δ*crp* Δ*cya*A (Table 1). Both W3110 *crp* and W3110 *crp cya*A strains of *E. coli* were proved to be Mal^- (white colonies) in MacConkey-Maltose plates (not shown).

7.2 Design of expression vectors for *crp* and *cya*A genes of *E. coli* and *P. putida*

The Δ*crp* and Δ*cya*A strains of *E. coli* W3110 described above were employed along with the cAMP-hypersensitive strain *E. coli* TP610A (Hedegaard & Danchin, 1985) for complementation of the cAMP-Crp dependent maltose consumption phenotype of *E. coli* in MacConkey agar plates as explained in the text. The plasmids used in these experiments were constructed as follows. In order to create a control *cya*A^+ plasmid, the *cyaA* coding region of *E. coli* was amplified along with 500 bp upstream and 200 bp downstream with primers aaECcyaA-F and aaECcyaA-R to ensure inclusion of its own native promoter and

terminator sequences. The flanking *Not*I sites generated with the same primers were then employed to clone the corresponding DNA segment (~3,260 bp) in *Not*I-digested pVTR-A vector (Table 2), thereby originating plasmid pARC2, which expresses $cyaA_{E.coli}$ through its native transcription and translation signals. A second *cyaA*$^+$ control plasmid was engineered to express the catalytic domain of the adenylate cyclase of *Bordetella pertussis* (i.e. residues 1 to 399 of its AC protein). The corresponding DNA segment was amplified from plasmid pDIA5240 (Ladant *et al.*, 1992) using primers aaBPcyaA-F and aaBPcyaA-R, which add terminal *Eco*RI and *Hin*dIII restriction sites to the amplified sequence. The resulting fragment of ~1.2 kb was then digested with these enzymes and cloned into the matching sites of IPTG-inducible expression vector pVLT31, giving rise to pARC3. To complete this set of expression vectors, the pMZC5 (low copy number) and pMZC6 (high copy number) were used as test plasmids encoding the adenylate cyclase of *P. putida* under the control of P*tac* and P*lac* promoters, respectively (Milanesio *et al.*, 2011).

To complete the entire set of complementation experiments, a $crp_{P.putida}$-expression plasmid was constructed as well. In this case, primers vfrMyc1 and vfrHis-C were used to amplify the promoterless $crp_{P.putida}$ coding region adding a 6xHis sequence at its 3' end, being the whole segment flanked by *Eco*RI-*Hin*dIII sites. The corresponding segment was then ligated to pUC18Not as an *Eco*RI/*Hin*dIII insert (originating pARV1) and subsequently recloned in expression vector pVLT31, generating plasmid pARV2. Additionally, a broad host range plasmid expressing the $crp_{E. coli}$ was constructed anew by recloning the 731 bp *Eco*RI-*Pst*I insert of pCA24N*crp* (Mori *et al.*, 2000) in vector pVLT31 (de Lorenzo *et al.*, 1993). The resulting construct, named pARCrp10, expresses a 6xHis-tagged version of $crp_{E. coli}$, which is functionally indistinguishable from the tag-less version. Other relevant plasmids used for complementation assays are listed in Table 2.

8 *Dictyostelium discoideum* cAMP chemotaxis biosensor

To examine whether *E. coli* or *P. putida* could secrete compounds able to elicit a chemotactic response in the slime mold *Dictyostelium discoideum* (Saran *et al.*, 2002) cultures of *E. coli* W3110 and *P. putida* MAD1 were prepared in M9 minimal medium with 0.2% fructose as C source and let grow overnight to ensure complete consumption of

the C source. Cells were then spun down, the supernatants filtered through a 0.45 μm pore membrane and the filtrates concentrated 5X in a Speed Vac. Additionally, starvation was induced in *D. discoideum* by first growing the mold in nutrient medium and then resuspending the cells for 16 hours in QQ2 phosphate buffer (KH_2PO_4 16.5 mM; K_2HPO_4 3.9 mM; pH 6.2) devoid of nutrients. The suspension of *D. discoideum* was then adjusted to a concentration of 2.5 x 10^8 cells/ml, and 1 μl of the culture was placed in a QQ2-Agar plate. To induce chemotaxis, 1 μl of the concentrated supernatants of *P. putida* and *E. coli* were placed in the QQ2-Agar plate at 4 mm distance from the *D. discoideum* spot (1 μl of starvation-induced culture). The plates were then incubated in a humid chamber for 3 hours at 22°C, after which formation of dendritic shapes towards the attractant at the rim of the *D. discoideum* population was considered an indication of chemotaxis and recorded with a Leica MZFLIII stereoscope.

9 Quantification of cAMP produced by *E. coli* and *P. putida*

In order to quantify the intracellular levels of cAMP in *E. coli* and *P. putida* and to measure the concentrations of this compound in the medium, the wild-type and *cya*A strains indicated in each case were grown on M9 minimal medium with 1% glycerol as sole C source (excepting *E. coli* Δ*cya*A, which was grown on glucose) until late exponential phase. The biomass of 10 ml of each of the samples was then collected by fast centrifugation (14000 rpm, 30 sec) and the pellets immediately frozen in liquid N_2 to quench the cell metabolome (Hajjaj *et al.*, 1998). cAMP was extracted from cells by vortexing the biomass with 25 mM ammonium acetate buffer (pH 7.2) in 60% ethanol at 70°C for 1 min, followed by incubation another 1 min at 70°C as described previously (Fuhrer & Sauer, 2009). The supernatants were pooled, dried in the Speed Vac and resuspended in 100 μl of MilliQ water. For quantification of extracellular cAMP, the supernatants of 10 ml cultures, were likewise frozen, lyophilized and resuspended in 0.5 ml of water. cAMP standards were prepared ranging 0.1-10 μg/ml to 10 μg/ml, their concentrations being accurately determined with a Varian Prostar 210 high-performance liquid chromatography system coupled to a Varian 1200L Triple Quadrupole MS. cAMP analyses were made following the Klawitter method (Klawitter *et al.*, 2007). To this end, samples were run on a Aqua C18 125A column (250 mm × 4.6 mm × 5 μm; Phenomenex), using a 50% mixture of

methanol and an aqueous buffer containing 10 mM dibutylamine, 15 mM ammonium acetate and 5% methanol as the mobile phase at a flow-rate of 0.5 ml/min. The injection volume was 10 µl and the HPLC-ESI-MS analysis was performed in the negative-ion mode.

10 Protein purification

10.1 Production and purification of MBP-Crp*P. putida* fusion protein

Preparation of Crp*P. putida* for detailed *in vitro* characterization required the implementation of a dedicated expression platform to produce it in large amounts, as an homogenous species and in a fully active form. To this end, the *crp* gene of *P. putida* KT2440 was amplified from genomic DNA with oligonucleotides aaMBPVfr-F and aaMBPVfr-R, which enter *Eco*RI and *Hind*III restriction sites to the 5' and 3' ends of the corresponding coding sequence, respectively. The resulting PCR fragment was cloned into pGEM-T cloning vector and the insert verified by DNA sequencing, thereby generating plasmid pARCrp4. In parallel, pMAL-C2T plasmid was then digested with *Eco*RI and *Hind*III to ligate the *crp*$_{P.\ putida}$-bearing segment of pARCrp4 bound by the same sites, what resulted in plasmid pARCrp5. Owing to its pedigree, this construct bears an in-frame fusion of *malE* (encoding the maltose binding protein, MBP) and *crp*$_{P.\ putida}$ genes separated by 18 nucleotides that encode the thrombin target sequence Leu-Val-Pro-Arg-Gly-Ser (Arce-Rodriguez *et al.*, 2012). To produce the MBP-Crp fusion protein, plasmid pARCrp5 was transformed into *E. coli* W3110 Δ*crp*. Transformants were inoculated in 800 ml of LB and the culture grown at 37°C to an OD_{600} = 0.5. At that point, 0.5 mM IPTG was added and the incubation continued for 2 hours for inducing expression of the plasmid-encoded protein. Cells were spun down and resuspended in 50 mM Na phosphate buffer pH 7.0, 200 mM NaCl and 1X of a protease inhibitor cocktail (Roche Applied Science), followed by disruption in a French press (Thermo, Electron corporation). Soluble extract was separated from cellular debris by centrifugation and then passed through an amylose-resin column (New England Biolabs). After washing the resin with 15 volumes of the same buffer, the recombinant MBP-Crp fusion protein was eluted by with the same solution as above containing 10 mM maltose. The MBP-Crp eluted fractions were analysed by SDS-PAGE (see below), and those which produced an apparently homogeneous protein band

were pooled, concentrated and its containing buffer was changed to 50 mM Na phosphate Buffer pH 7.0, 200 mM KCl, 20% (v/v) glycerol using a 50 kDa molecular weight-cutoff centrifugal filter device (Millipore). Final concentration of the thereby prepared MBP-Crp$_{P. putida}$ was 7.1 mg/ml, as determined by Bradford assay using BSA as standard (Sigma-Aldrich).

10.2 Cleavage of MBP-Crp and purification of Crp$_{P. putida}$

For purification of native-size Crp$_{P. putida}$, 16 mg of the MBP-Crp$_{P. putida}$ fusion protein were first dialyzed against PBS for 8 hours at 4°C, followed by cleavage with 170 U of thrombin protease (Amersham Biosciences) for 15 hours at 22°C in a volume of 5 ml. The reaction mixture was then passed during 6 hours through 1.6 ml of P11 phosphocellulose resin column (Whatman) previously equilibrated with PBS, with a constant flux of 0.5 ml/min. The resin was extensively washed with 35 ml PBS for eluting the MBP moiety away of the column-bound MBP-less Crp product. After such an elution was complete, the Crp$_{P. putida}$ protein was recovered by washing with the same buffer with 500 mM KCl. The Crp$_{P. putida}$ – containing fractions were pooled, concentrated in a 10,000 MWCO centricon device (Millipore) and dialyzed as before against 50 mM Na phosphate buffer pH 7.0, 250 mM KCl, 0.67 mM DTT, 25% (v/v) glycerol and finally stored at -80°C. Purified Crp protein was > 95% pure as determined by SDS-PAGE, and its concentration determined to be 1 mg/ml by RC DC Protein Assay (Bio-Rad). To verify the integrity of the purified protein, the molecular mass of Crp$_{P. putida}$ was determined by mass spectrometry using a PCS-4000 SELDI-TOF platform (BioRad).

10.3 Production and purification of apo-Crp$_{P. putida}$

To purify large amounts of cAMP-less apo-Crp$_{P. putida}$, we introduced several modifications to the protocol described above. First, the pARCrp5 was transformed in the *E. coli* W3110 Δ*crp* Δ*cya*A double mutant (instead of the Δ*crp* strain) and this strain was grown in 800 ml of M9-glucose minimal medium to an OD_{600} = 0.5. This avoids the production cAMP by the cells and the presence of this metabolite in the culture media. Then, the cells were induced, collected and disrupted following the earlier protocol, and the cAMP-less MBP-Crp$_{P. putida}$ was purified using the amylose-resin column like previously. Approximately 12 mg of apo-MBP-Crp were extensively dialyzed against PBS during 8 hours at 4°C. Prior to

cleaving, 0.5 mM DTT was added to the fusion protein and then it was overnight digested with 240 U of thrombin at 22°C. Immediately after cleavage, the protein mixture was passed through 5 ml of PBS-equilibrated P11 phosphocellulose resin column (Whatman) and washed extensively with 80 ml of PBS. Elution of purified $Crp_{P.\ putida}$ was achieved by washing the resin with PBS + 500 mM KCl. The eluted fractions were pooled, concentrated and dialyzed against 50 mM Na phosphate buffer pH 7.0, 250 mM KCl, 0.67 μM DTT and 20% (v/v) glycerol. The concentration of the protein was calculated to be 0.8 mg/ml as determined by absorbance at 280 nM, using a calculated extinction coefficient of 11.46 x 10^3 M^{-1} cm^{-1} (ProtParam tool, ExPASy server; Gasteiger *et al.*, 2005).

11 Analytical ultracentrifugation of $Crp_{P.\ putida}$

The effects of cAMP in the oligomerization state of Crp were examined in an XLI analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with a UV-visible absorbance detection system. *Sedimentation velocity* experiments were performed in an An-50Ti rotor at 20°C and 48 krpm loaded with samples of $Crp_{P.\ putida}$ at a concentration of 49.6 μM prepared in 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl and 102.7 mM KCl (pH 7.4), with or without 1 mM cAMP. Sedimentation profiles were recorded every 3 min at 285 nm. The corresponding sedimentation coefficient distributions c(s) were calculated by least squares boundary modelling of sedimentation velocity data using SEDFIT v12.1 software (Schuck, 2000). From these analysis, the experimental sedimentation coefficients were corrected to standard solvent conditions (pure water, 20°C, and infinite dilution) using the SEDNTERP program (Laue *et al.*, 1992) for generating the corresponding standard-values ($S_{20,W}$). *Sedimentation equilibrium* experiments were made in the same instrument under identical Crp concentration and buffer conditions as the *sedimentation velocity* assays before, but at a lower temperature (10°C) in order to prevent formation of protein aggregates. In this case, measurements were taken at 11 and 14 krpm using short ultracentrifugation columns with 85 μl of protein sample. After the equilibrium scans, a high-speed centrifugation run (43 krpm) was done to estimate the corresponding base-line offsets. The corresponding buoyant signal average molecular weights of the samples were determined by fitting the experimental data to the equation that characterizes the equilibrium gradient of an ideally sedimenting solute using Hetero-Analysis software (Cole,

2004). The average molecular weight of Crp was determined from the experimental buoyant molecular weight values using the partial specific volume (0.733 ml/g) calculated from the amino acid composition using SEDNTERP (Laue *et al.*, 1992).

12 Partial proteolysis of Crp*P. putida*

For partial proteolysis assay, 3.8 μg of the pure Crp were incubated in a 20 μl reaction mixture containing 50 mM Na phosphate buffer pH 7.0, 250 mM KCl and (where indicated) 1 mM cAMP for 10 minutes at 22 °C. Digestion reactions were started by addition of 30 ng of proteomics grade trypsin (Sigma-Aldrich), followed by incubation for 0-45 min at 37°C. Proteolysis was stopped by addition of 5 μl of 5X protein loading dye [300 mM Tris-HCl pH 6.8, 5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue]. Samples were boiled 5 min at 100°C, electrophoresed in a 15% SDS-polyacrylamide gel and the products stained with Coomasie blue as described before.

13 Isothermal titration calorimetry (ITC)

Initially, the isothermal titration calorimetry experiments were performed with the MBP-Crp*P. putida* fusion protein as follows. Prior to experiments, the MBP-Crp*P. putida* solution was exhaustively dialyzed in 50 mM K phosphate buffer pH 7.0, 150 mM KCl and 20% (v/v) glycerol. Next, the protein was passed through a 0.22 μm pore size filter and its concentration was accurately determined by UV absorption spectroscopy using a calculated extinction coefficient of 7.8 x 10^4 M^{-1} cm^{-1} at 280 nm (ProtParam tool, ExPASy server; Gasteiger *et al.*, 2005). Putative effector molecules were prepared by dissolving pure compounds (cAMP, cGMP and AMP) in filtered dialysis buffer described above to homogenize ligand and protein solvent. MBP-Crp*P. putida* was diluted to 10 μM and 1.4 ml of protein was titrated in a VP microcalorimeter (MicroCal, Northampton, MA, USA) at 25°C. First titration was carried out with a single 1.6 μl injection of diluted ligands at 350 μM, followed by subsequent injections with 4.8 μl of the same compounds. The mean enthalpies measured from injection of the ligands into the protein-less buffer were

subtracted from raw titration data. The mean enthalpies measured from the injection of ligand into buffer were subtracted from the raw titration data, prior to curve fitting using the *One Binding Site* model of the MicroCal (Northampton, MA, USA) version of ORIGIN. From the values obtained for K_A and enthalpy (ΔH), the dissociation constant K_D was determined ($K_D = 1/K_A$) as well as the changes in the free energy (ΔG) and entropy (ΔS) using the equation ($\Delta G = -RT\ln K_A = \Delta H - T\Delta S$, where R is the universal molar gas constant and T the absolute temperature.

Additionally, ITC experiments with purified Crp$_{P.\ putida}$ devoid of cAMP (apo-Crp$_{P.\ putida}$) and the cyclic nucleotides (cAMP and cGMP) were carried out on the basis of the previous procedures, but changing the salt concentration of the reaction buffer to 250 mM KCl (in the case of the interaction with cAMP) and incorporating 1 mM DTT to the reactions. This modification was incorporated in order to stabilize the protein in solution and provide a reduced environment for its Cys residues. The titrations were conducted with 8-7 μM of apo-Crp$_{P.\ putida}$ and 200 μM of cyclic nucleotides, following the same protocol described above.

14 Electrophoretic mobility shift assays (EMSA)

To prepare a DNA fragment suitable for gel retardation experiments a 330 bp sequence containing the *lac* promoter (P*lac*) of *E. coli* was amplified from plasmid pUC18Not (Herrero *et al.*, 1990) with primers aaPlac-F and aaPlac-R. The resulting DNA product, that retains the *Eco*RI site of pUC18Not polylinker, was digested with this restriction enzyme and 3'-end labelled by filling the overhanging end of the cleaved site with [α-^{32}P] dATP and Klenow DNA polymerase (Sambrook *et al.*, 1989). Binding reactions were performed in 10 μl of TRRG buffer [20 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM β-mercaptoethanol, 10% (v/v) glycerol] containing 0.5 ng end-labelled P*lac* probe, 5 μg BSA, 5 ng poly deoxyinosinic-deoxycytidylic acid (poly dI-dC) and increasing amounts of Crp ranging 10-250 nM. Where indicated, 100 μM of cAMP or 100 μM cGMP were added to the reactions. Samples where incubated 20 minutes at 30°C and electrophoresed in a 4% non-denaturing polyacrylamide gel prepared in 0.5X TBE buffer (Sambrook *et al.*, 1989).

DNA band shifts were observed by autoradiography of the dried gels on X-ray film (Konica Minolta).

15 *In vitro* transcription assays (IVT)

For preparation of a supercoiled DNA template for the *in vitro* transcription (IVT) experiments, the *lac* promoter (which has the $Crp_{E.\ coli}$ binding site 5'TAATGTGAGTTAGCTCACTCAT3' centered around position -61.5) was amplified as a 391 bp fragment from pUC18Not (Herrero *et al.*, 1990) with primers 5'-PlacIVT and 3'-PlacIVT, leaving the region of interest flanked by an upstream *Sca*I blunt restriction site and a downstream *Eco*RI (this latter restriction site is within the pUC18Not polylinker). This was then cloned into vector pJCD01 previously digested with *Sma*I and *Eco*RI, resulting in plasmid pJCD-Plac (Table 2), which was employed as DNA template in all subsequent *in vitro* transcription experiments. IVT cocktails were set in 50 µl volumes containing 50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM BSA, 10 mM DTT, 1 mM EDTA, 30 nM of purified *Pseudomonas putida* RNA polymerase holoenzyme prepared as described in Johansson *et al.* (2008); the kind gift of C. Alvarez), 5 nM of pJCD-Plac and Crp ranging 10-100 nM as specified. Where indicated, such a mixture was pre-incubated at 30°C for 10 minutes with 0.5 mM cAMP or cGMP prior to transcription start. Transcription was initiated by adding a cold ATP, CTP, and GTP 500 µM (each) and UTP 50 µM premixed with 2.5 µCi of [α-^{32}P]UTP (3,000 mCi/mmol). Following a 15 min incubation of the samples at 30°C, transcription reactions were stopped with 50 µl of a STOP mixture 50 mM EDTA, 350 mM NaCl, and 0.5 mg/ml of carrier tRNA. mRNA was precipitated with absolute ethanol at -20°C and resuspended in loading buffer containing 7 M urea, 1 mM EDTA, 0.6 M glycerol, 0.9 mM bromophenol blue and 1.1 mM xylene cyanol. The resulting samples were electrophoresed on a denaturing 7 M urea–4% polyacrylamide gel, and visualized by autoradiography.

16 β-galactosidase assays

16.1 Construction of plasmids encoding *lacZ* transcriptional and translational fusions

To measure the activity of $crp_{P.\ putida}$ promoter (P*crp*), a *lac*Z translational fusion was generated by PCR amplification of the 400 bp upstream the start codon (ATG) of *crp* plus the DNA sequence codifying its 30 first amino acids. PCR was conducted with oligonucleotides aaPcrp-F and aaPcrpTrad-R, which enter *Eco*RI and *Bam*HI restriction sites. The corresponding 492 bp fragment was digested and cloned into the same sites of pSEVA225T, thereby generating the in-frame fusion of Crp (30 first aa) with LacZ. The resulting plasmid was named pARPcrp1. Following this line, a translational fusion of $cyaA_{P.\ putida}$ promoter (P*cya*A) was created by PCR amplification of a 492 bp DNA fragment that includes the 400 bp upstream the ATG of *cya*A gene plus the sequence of its 30 first codons. The reaction was carried out with oligonucleotides aaPcyaA-F and aaPcyaATrad-R, which enter *Eco*RI and *Bam*HI sites respectively. The resulting fragment was *Eco*RI/*Bam*HI digested, and cloned in the same sites of pSEVA225T to create pARPcyaA1. Finally, in order to compare the transcription and translation of $cyaA_{P.\ putida}$ promoter, a transcriptional fusion of this gene was constructed by PCR amplifying the 400 bp upstream the *cya*A start codon, using oligonucleotides aaPcyaA-F (*Eco*RI) and aaPcyaA-R (*Bam*HI). The resulting ~0.4 kb fragment was EcoRI/BamHI digested and subsequently cloned in the pSEVA225T previously cleaved with the same restriction nucleases, generating pARPcyaA2. All these plasmids were transformed in the *P. putida* strains KT2440 wt, KT2440 Δ*crp* and KT2440 Δ*cya*A.

16.2 Measurements of the β-galactosidase activity

The LacZ activity of the P*crp translational fusion* was analysed as follows. First, a single colony of the different *P. putida* strains transformed with pARPcrp1 was grown in 10 ml of M9 minimal medium supplemented with Km and 0.2% (w/v) of the carbon source indicated in each case (glucose, fructose or succinate). The cells were cultured until exponential growth phase and then, a small aliquot was obtained to measure the β-galactosidase activity according to the classical method described by Miller (1972). The same protocol described above was initially employed to measure the β-galactosidase activity of the P*cya*A *translational* fusion, however the enzymatic values obtained were too low. Thus, we decided to measure the LacZ activity of P*cya*A *translational* and

transcriptional fusions with the ultra-sensitive Galacto-Light Plus™ commercial system (Applied Biosystems). According with manufacturer specifications, this method incorporates a chemiluminiscent substrate for β-galactosidase and luminescence enhancers that make the system more sensitive to low amounts of enzyme. The methodology employed was as follows: one single colony of the different *P. putida* strains (transformed either with pARPcyaA1 or pARPcyaA2) was cultured in 10 ml of M9 minimal medium supplemented with Km and 0.2% (w/v) of carbon source. When the cultures reach exponential phase, 500 µl of cells were taken into an Eppendorf tube and pelleted by centrifugation 2 min at 14,000 rpm. The bacteria were then resuspended in 200 µl of lysis buffer [100 mM potassium phosphate pH 7.8, 0.2% (v/v) Triton X-100], subjected to two freeze-thaw cycles and centrifuged 1 min at 14.000 rpm to separate the cell debris. 20 µl of lysed supernatant were incubated with 80 µl of reaction buffer (100 mM sodium phosphate pH 8.0, 1 mM $MgCl_2$ and 1X Galacto-Plus® chemiluminiscent substrate) previously distributed in 96-well plates. The reaction was incubated in the dark for 30 minutes, after which 125 µl of Sapphire-II™ light emission accelerator were added. The reactions were incubated 1 min in the dark and finally the luminescence was recorded in the Victor 2 multireader spectrophotometer (Perkin Elmer).

17 RNA-seq transcriptome analysis of *crp* and *cya*A mutants in *P. putida*

17.1 Culture conditions, RNA isolation and rRNA removal

An overnight culture of *P. putida* strains KT2440 and its *crp* and *cya*A mutants were grown in M9 medium supplemented with 0.2% glucose as carbon source. A 1/100 dilution of this pre-culture was used to inoculate 10 ml of the same medium with each strain. Bacteria were grown to mid-exponential phase (OD_{600}~0,4-0,5) and then, transcription was halted by adding 1.25 ml of Stop solution [5% (v/v) saturated phenol dissolved in pure ethanol]. The samples were transferred to a Falcon tube and after vortexing, cells were spun down by centrifugation 20 min at 4000 and 4°C. Next, the cell pellets were resuspended in 300 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH8) containing 2 mg/ml lysozyme, followed by incubation at 37°C for 10 min. 100 µl of cell lysates were transferred to an Eppendorf tube and RNA isolation was carried out using the miRNeasy kit from QIAGEN

(Cat. Nº 217004) according to manufacturer specifications. RNA concentration was checked in the NanoVue spectrophotometer (GE Healthcare) and the quality of the RNA samples was assessed by running a small aliquot on the 2100 Bioanalyzer (Agilent Technologies). Further removal of 23S, 16S and 5S ribosomal RNA (rRNA) was performed with Ribo-Zero™ rRNA Removal Kit (Epicentre; Cat. Nº RZNB1056), following manufacturer protocols.

17.2 RNA-Seq using the Illumina Genome Analyzer

Samples were prepared in accordance with the *Illumina* RNA preparation protocol (Part # 1004898 Rev. A from September 2008) by the sequencing company BGI (Hong-Kong, China; http://www.bgisequence.com/eu/). The experimental pipeline of the RNA sequencing protocol is shown in Fig. 5. Briefly, the rRNA-free mRNA was chemically disrupted in shorter fragments by adding Fragmentation Buffer. Taking these cleaved RNA fragments as templates, random hexamer-primers and reverse transcriptase were used to synthesize the first-strand of cDNA. Following, the second-strand of cDNA was synthesized using GEX Second Strand Buffer, dNTPs, RNase H and DNA polymerase I. Short cDNA fragments were purified with QiaQuick PCR extraction kit and resolved

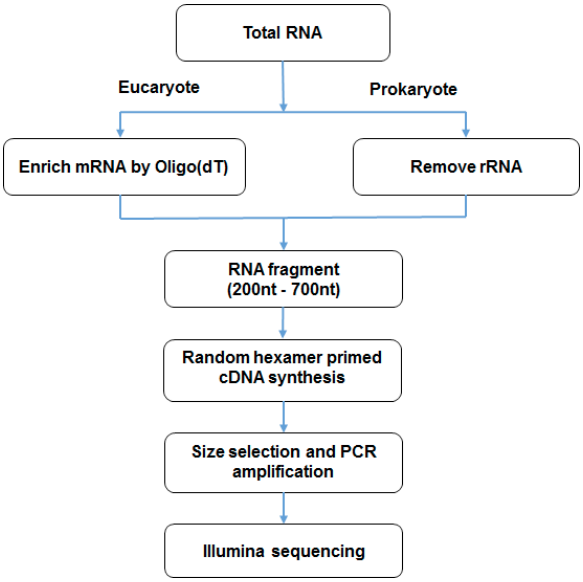


Fig. 5. Pipeline of the RNA-seq protocol for transcriptome analysis using the *Illumina* sequencing platform. BGI company (Hong-Kong).

with EB buffer to perform the end-repair protocol. This method converts the overhangs into blunt ends using T4 DNA polymerase, Klenow DNA polymerase and dNTP mix. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs. Next, addition of 'A' bases to the 3' end of the blunt phosphorylated DNA fragments was achieved using the polymerase activity of Klenow enzyme. 'A'-tailing prepares the DNA fragments for ligation to the adapters (using T4 ligase), which have a single 'T' base overhang at their 3' end. After adapters adhesion, total DNA was run on an agarose gel to select a size range of fragments. DNA of suitable size (approximately 200 bp) was purified and used as template for downstream enrichment, carried out by PCR reaction with two primers that anneal to the ends of the adapters. Finally, after validation of the DNA size with Bioanalyzer (Agilent Technologies), the library was sequenced using the Illumina HiSeq™ 2000 apparatus.

17.3 Bioinformatic analysis

Images generated by sequencers were converted into nucleotide sequences by *base calling*, thereby generating the raw data (stored in FASTQ format). Nevertheless, the information contains reads from the adapters, unknown nucleotides or low quality reads that could negatively affect the following bioinformatics analysis. Therefore, these *dirty reads* were discarded to generate the clean data. Once filtered, the clean reads were mapped to *P. putida* KT2440 reference genome and to reference gene sequences using SOAP2 (Li *et al.*, 2009). Proportions of clean reads mapped to the genome and to individual genes provided an overall assessment of the sequencing. The expression of annotated genes was evaluated with two parameters. First, the percentage of each gene covered by reads (or gene coverage) was calculated. This value equals the ratio of the number of bases in a gene covered by unique mapping reads to number of total bases in that gene. Second, the expression of individual genes was calculated as RPKM (Reads Per kb per Million mapped reads; Mortazavi *et al.*, 2008) using the following formula:

$$\mathrm{RPKM} = \frac{10^6 C}{NL/10^3}$$

The RPKM value measures the expression of a gene as a function of the number of reads that uniquely align to that gene (C), divided by the total number of reads that align to all

genes (N) and the number of bases in the CDS of the evaluated gene (L). The RPKM method eliminates the influence of gene length and sequencing discrepancy over the calculation of gene expression. Therefore, the calculated gene expression can be directly used for comparing the difference of gene expression among samples.

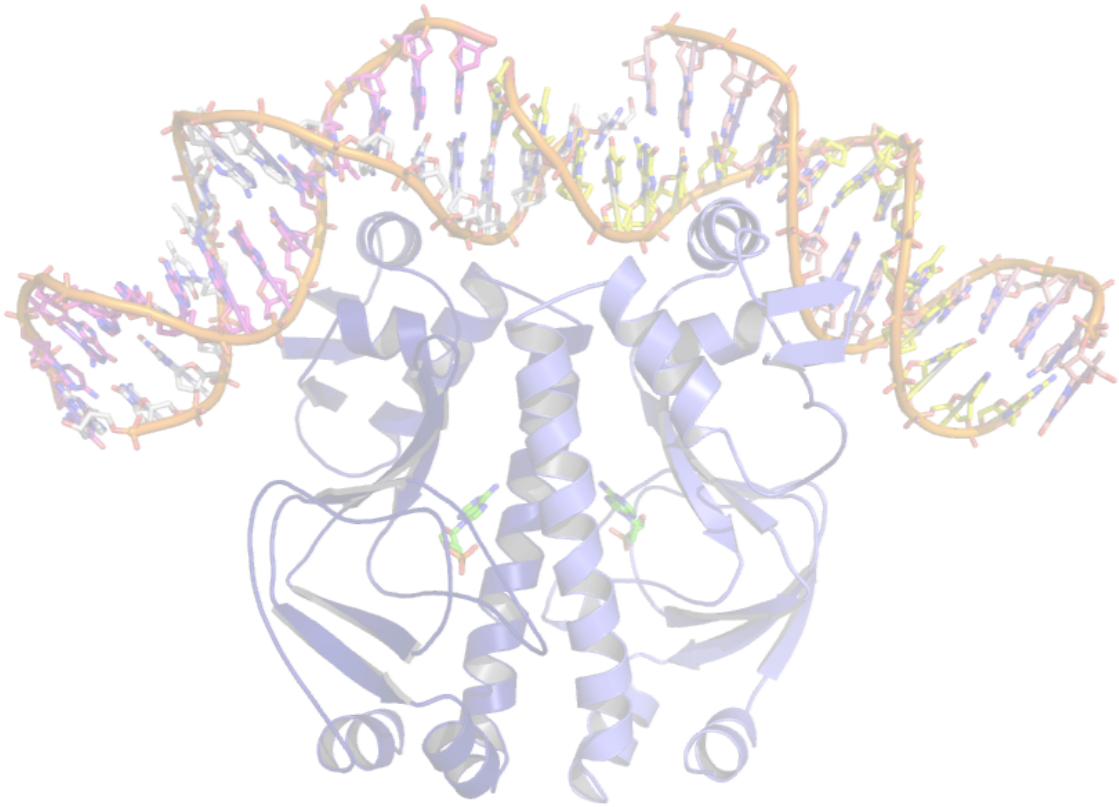
17.4 Estimation of Differential Expression

The identification of differentially expressed genes between two samples was carried out with a rigorous algorithm that calculates the *p*-value on the basis of reference *The significance of digital gene expression profiles* (Audic S, Genome Research). Additionally, the FDR (False Discovery Rate) was calculated as a statistical method to test and correct the results obtained with the p-value. In practical terms, FDR is the expected false discovery rate between the differential expression of every gene (Benjamini & Hochberg, 1995). The smaller the FDR value and the larger the fold change ratio between two samples, the greater the difference in the gene expression between them will be. In our analysis, we filtered the differentially expressed genes with FDR ≤ 0.001 and fold change ratio larger than 2.8, using the FIESTA v1.0 viewer (Oliveros, 2007). Positive fold change values represent the upregulated genes in the Δ*crp* and Δ*cya*A strains with respect to the wild-type *P. putida* KT2440 reference, while negative values represent the downregulated genes of the mutants respective to wild-type strain.

IV. Results

Chapter I

Genetic and phenotypic characterization of the components involved in the cAMP-Crp system of *Pseudomonas putida*



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Milanesio, P., A. Arce-Rodriguez, A. Munoz, B. Calles & V. de Lorenzo, (2011) Regulatory exaptation of the catabolite repression protein (Crp)-cAMP system in *Pseudomonas putida*. *Environ Microbiol* **13**: 324-339.

1 The cAMP-Crp system of *Pseudomonas putida* and its components.

The genome of *P. putida* KT2440 encodes singular orthologues of the *cya*A and *crp* genes of *E. coli*, which are respectively annotated with the gene loci PP_5222 and PP_0424 (Nelson *et al.*, 2002), http://cmr.jcvi.org). By looking on its genome context, it is noteworthy that both $crp_{P.\ putida}$ and $cyaA_{P.\ putida}$ occur as stand-alone genes, and therefore, it is likely that they are expressed as a single cistron and thus unrelated to the adjacent ORFs (Fig. 6A and Fig. 6B). To complete this picture, a third partner of the system was recently characterized in *Pseudomonas aeruginosa*. This is the CpdA protein, a class III phosphodiesterase encoded by the *cpd*A gene that catalyses the hydrolysis of cAMP to 5'-AMP (Fuchs *et al.*, 2010b). In order to find a similar gene (or genes) codifying a putative phosphodiesterase in *P. putida* strain KT2440, we searched in the genome of this bacterium for genes whose protein products were similar to the CpdA of *P. aeruginosa* (PA4969) using the protein BLAST tool of the NCBI (http://blast.ncbi.nlm.nih.gov). Only one protein of 266 aa residues results to be highly similar to $CpdA_{P.\ aeruginosa}$, with 54% identity and 69% similarity. This positive match was previously annotated as the Icc protein, encoded by the gene locus PP_4917 (Fig. 6C; Nelson *et al.*, 2002). Unlike *crp* and *cya*A, that are transcribed as stand-alone genes, *cpd*A is co-transcribed in a large operon that include the *par*C and *par*E genes, encoding respectively the A and B subunits of a type IV DNA topoisomerase. We aligned the amino acid sequence of the Icc protein with the homologous phosphodiesterases of *E. coli* and *P. aeruginosa* and we find that the 13 residues that are absolutely conserved in all the class III phosphodiesterases are also present in the protein encoded by PP_4917 (Figure 6D). Yet, given to the sequence analysis, we decided to rename this protein as $CpdA_{P.\ putida}$ that stands for cAMP-phosphodiesterase.

Despite the similarity of $crp_{P.\ putida}$ and $cyaA_{P.\ putida}$ with the respective orthologues of *E. coli*, these genes have not been related with any catabolite repression phenomena (Rojo, 2010). Therefore, we decided to construct full deletion mutants of *crp* and *cya*A in the *P. putida* strain KT2440 to decipher whether the loss of these genes causes a perturbation in the metabolism of carbon sources in this bacterium. In addition, a mini-Tn*5* insertion mutant in the *cpd*A phosphodiesterase was obtained from the *P. putida* KT2440 collection (Duque *et al.*, 2007). These strains, together with wild-type KT2440, were grown on M9 minimal medium with three different carbon sources, covering a considerable range of metabolic

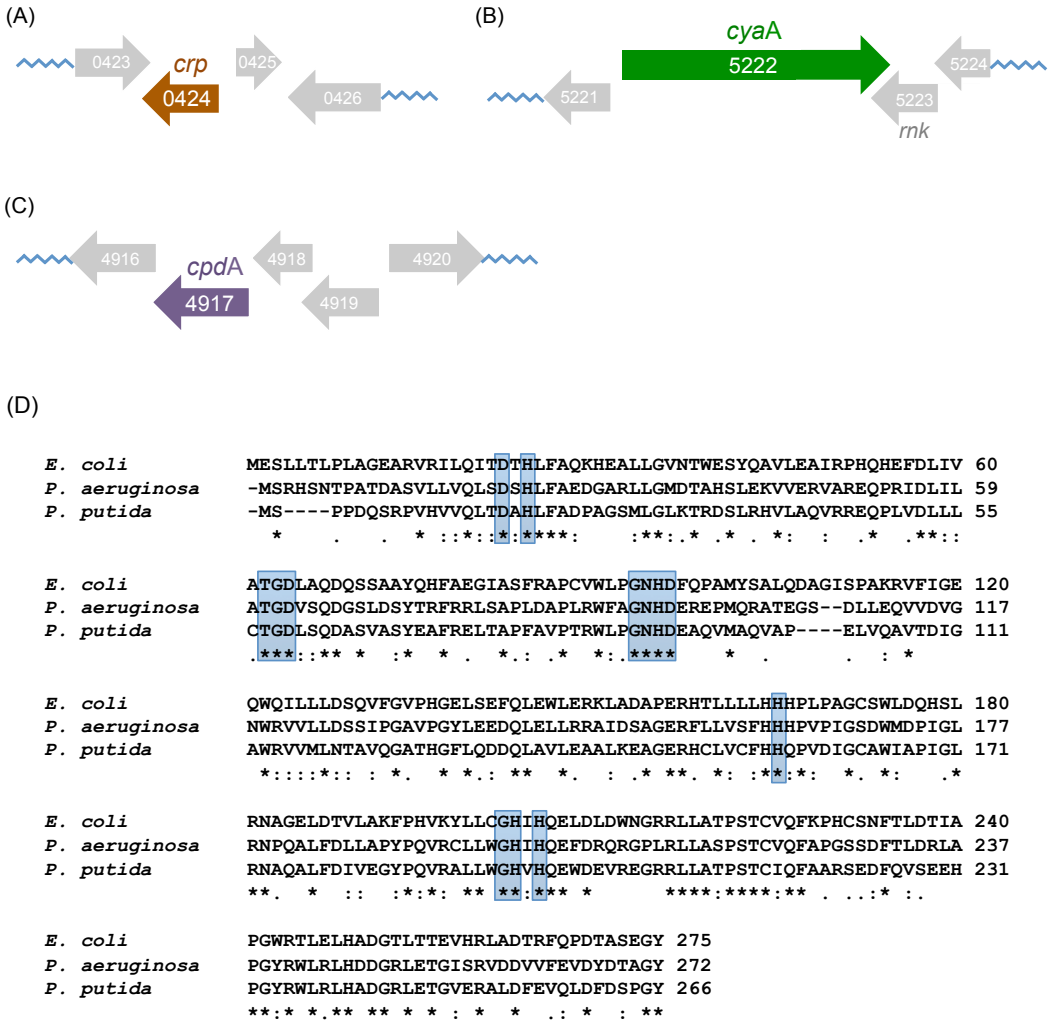


Fig. 6. Components of the cAMP-Crp system of *P. putida* KT2440. The upper panel shows the genome context of **(A)** *crp* (PP_0424), **(B)** *cya*A (PP_5222) and **(C)** *cpd*A (PP_4917). Note that *crp, cya*A and *cpd*A map in very different locations of the *P. putida* chromosome. While *crp* and *cya*A are transcribed as stand-alone genes, *cpd*A is co-transcribed with other genes in a large operon. **(D)** Alignment of CpdA proteins from *E. coli* W3110, *P. aeruginosa* PAO1 and *P. putida* KT2440. The symbols below each alignment indicate fully conserved (*), strongly similar (:) and weakly similar (.) amino acids. The 13 residues framed in blue correspond to conserved aa between all the class III phosphodiesterases (Richter, 2002), all of which were conserved in the CpdA$_{P.\ putida}$ counterpart. The alignment was generated with the online ClustalW2 software (Larkin *et al.*, 2007).

conditions: on the one hand, succinate is a gluconeogenic substrate, while glucose is consumed through the Entner–Doudoroff (ED) pathway (del Castillo *et al.*, 2007). Fructose in turn is transformed into fructose-1,6-bisphosphate by the glycolytic

pathway, and then it can continue through glycolysis or be metabolized though the ED pathway (Velazquez *et al.*, 2004; Chavarria *et al.*, 2012).

By surveying the results shown in Fig. 7, the first noticeable feature is that there is virtually no difference in the growth rate between the wild-type strain and the Δ*crp* or Δ*cya*A mutants in the three carbon sources tested. This is, however, in accordance with similar results obtained by Daniels *et al.* (2010) and also with the phenotypic microarray developed by Milanesio *et al.* (2011), in which the mutation of both genes have no effect on the catabolism of carbon sources employed. Therefore, it seems that *crp* and *cya*A mutations have little, if any, influence in the general utilization of carbon sources in this bacterium. In contrast, the *cpd*A mutant shows a considerable growth rate decrease when it is grown on succinate. This was also evident, though a lesser extent, in the presence of glucose.

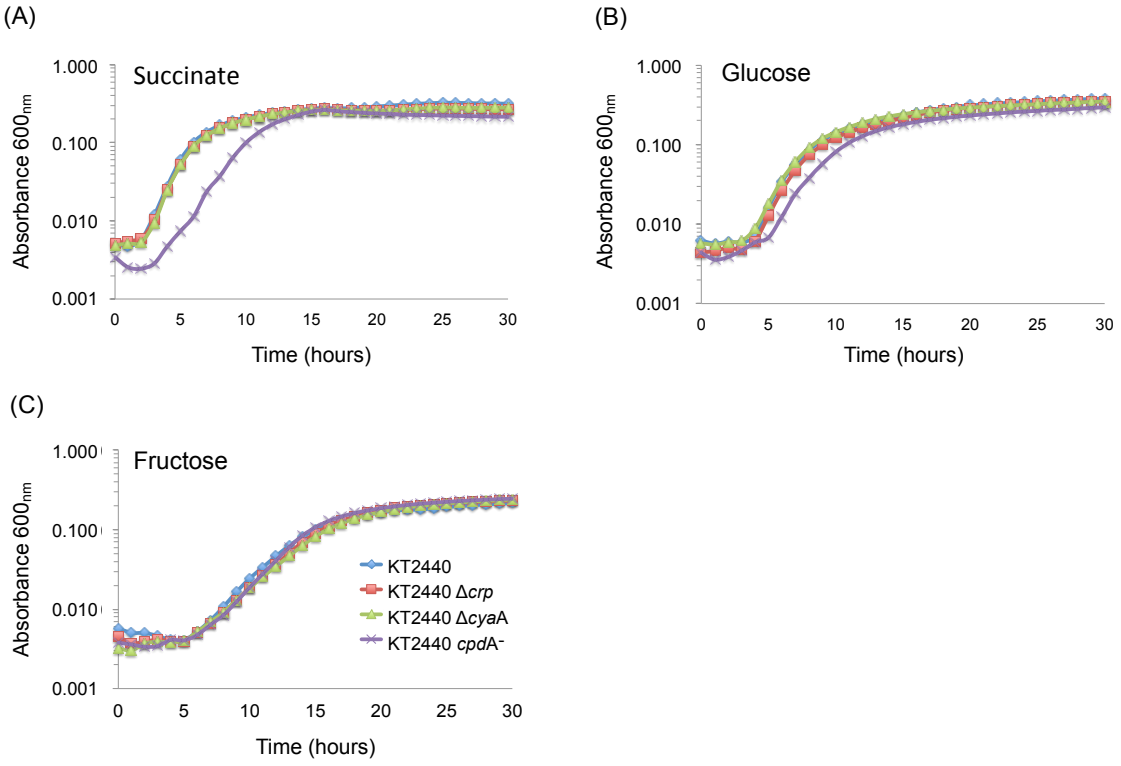


Fig. 7. Growth curves of cAMP-Crp system mutants on three different carbon sources. Triplicate cultures of *P. putida* KT2440 wild-type, Δ*crp*, Δ*cya*A and *cpd*A- strains were grown at 30°C in M9 medium supplemented with 0.2% (w/v) of the carbon substrate specified in each case: **(A)** Succinate; **(B)** Glucose and **(C)** Fructose. The OD_{600} was recorded each hour in microtiter plates with the Victor 2 multireader spectrophotometer (Perkin Elmer).

No effects were observed when the mutant grew on fructose. A similar behaviour was detected in a *cpd*A mutant of *P. aeruginosa* when grown on rich LB medium, but not on succinate or glucose. However, this phenomenon is not related with the increase of intracellular cAMP due to *cpd*A mutation and in contrast, it is attributed to another unknown function (Fuchs *et al.*, 2010b).

2 Functional characterization of *P. putida crp* and *cya*A gene products.

2.1 The *crp* gene of *P. putida* and its encoded transcriptional regulator

Fig. 6A shows the genomic context of the unique *crp* ortholog that can be found in the genome of *P. putida* KT2440 with the gene locus PP_0424 (http://cmr.jcvi.org). It is worth to mention that despite 62% of identity and 80% of similarity of the encoded product with the Crp protein of *E. coli* (Fig. 8), PP_0424 was not annotated as such in the primary analysis, but as the cyclic nucleotide-binding transcriptional regulator Vfr, due to the similarity with the Virulence Factor Regulator of *P. aeruginosa*. Moreover, as indicated in Fig. 8, $Crp_{P.putida}$ bears a considerable conservation of the functional traits of the *E. coli* counterpart. These include the helix-turn-helix (HTH) domain for DNA binding as well as a suite of residues needed for cyclic nucleotide monophosphate binding. Despite the conservation of amino acids, the differences in the residues observed in the HTH motif of the three different Crp orthologues, suggest that the DNA sequences bound by each of the proteins are likely to be similar, but not necessarily identical. It is also noteworthy that the only change observed in the cNMP interaction pocket of the $Crp_{P.putida}$ is a substitution of the Ser residue in position 128 of $Crp_{E.coli}$ for a Thr in the equivalent 133 amino acid of *P. putida*. This change is important, because a S128T exchange in $Crp_{E.coli}$ is known to make the mutant responsive to both cAMP and cGMP (Lee *et al.*, 1994; Youn *et al.*, 2008), raising the possibility that $Crp_{P.putida}$ uses both cAMP and cGMP as effectors. Additionally, the $Crp_{P.putida}$ sequence maintains the 3 regions identified in the *E. coli* protein responsible for interacting with various sites in the surface of the RNAP: AR1, necessary for activation of class I and class II promoters through direct contacts with the α-CTD; AR2, required for class II promoters via interactions with the α-NTD and the optional surface AR3, which

seems to interact with residues 590-600 of the region 4 of σ^{70} (Busby & Ebright, 1999; Zhou *et al.*, 1993; Zhou *et al.*, 1994; Parkinson *et al.*, 1996; Niu *et al.*, 1996; Rhodius & Busby, 2000a) . Taken together, all these similarities indicate that this protein is equivalent to the reference $Crp_{E.coli}$ transcription factor.

Fig. 8. Comparison between the Crp homologues in *E. coli*, *P. aeruginosa* and *P. putida*. The image shows the corresponding amino acid sequences along with an indication of the key functional ingredients as follows. : residues involved in cAMP binding (West *et al.*, 1994). : stabilization of cAMP binding (West *et al.*, 1994). : residues involved in Crp-RNAP interactions: AR1, AR2 and AR3 (activating regions; Niu *et al.*, 1996; Busby & Ebright, 1999; Zhou *et al.*, 1993). : Helix-turn-helix, DNA binding motif (Parkinson *et al.*, 1996; West *et al.*, 1994). : Residues that make direct contact with the DNA base edges (Parkinson *et al.*, 1996; West *et al.*, 1994). : Residues that make contact with the phosphates of DNA (Parkinson *et al.*, 1996). AR1 consist of the residues 156-164 of Crp. The side chain of Thr158 seems to be the most important for its function (it is conserved in *P. putida* and *P. aeruginosa*). AR1 is necessary for activation of class I and class II promoters through direct interaction with the α-CTD (Zhou *et al.*, 1993; Busby & Ebright, 1999). AR2 is a specific determinant consisting of residues His19, His21, Glu96 and Lys101, which are necessary for the activation of class II promoters. In both *P. putida* and *P. aeruginosa*, the His21 is substituted for an Arg. Moreover, His19 is substituted in *P. putida* by a Glu residue. AR2 is proposed to interact with the α-NTD after initial binding of the RNAP to promoter DNA (Niu *et al.*, 1996; Busby & Ebright, 1999). AR3 is the product of a non-native Crp-RNAP interaction at class II promoter and it is created by substitution of Lys52 by a neutral or negatively charged residue. Interestingly, both in *P. putida* and *P. aeruginosa* there is a Glu at position 52 that probably makes functional the AR3 determinant as well (Niu *et al.*, 1996; Busby & Ebright, 1999; Rhodius & Busby, 2000b)

2.2 *In vivo* functional complementation of Crp$_{E. coli}$ by Crp$_{P.putida}$

The comparison of the different Crp homologues described before suggests that Crp$_{P.putida}$ could operate in a similar fashion than its counterpart in *E. coli*. To verify this hypothesis, we examined the ability of *crp*$_{P.putida}$, expressed at various levels, to restore the Crp-dependent maltose utilization in *E. coli* strains with relevant genotypes (Fig. 9). The reference condition is that of row 1, in which *E. coli* W3110 Δ*crp* was transformed with a high-copy number plasmid (pCA24N*crp*, Table 2) which expressed *crp*$_{E.coli}$ under the control of a heterologous IPTG-inducible promoter. This strain was streaked along with the corresponding insert-less vector on the fermentation-indicative MacConkey medium supplemented with 1.0% (w/v) maltose. As shown in Fig. 9a (row 1), *crp*$_{E.coli}$ restored the Mal$^+$ phenotype even when expressed through the escape of the vector promoter in the absence of induction. Fig. 9a (row 1) also shows that IPTG addition was toxic to the host Δ*crp E. coli* strain. The situation was similar, but not identical, when *crp*$_{E.coli}$ was replaced by *crp*$_{P.putida}$ in the same test system (Fig. 9a, row 2). When cloned in a multicopy plasmid (pARV1), the gene from *P. putida* complemented the lack of *crp* in the host *E. coli* strain. Finally, when expressed from a low copy inducible vector (pARV2), IPTG addition was required to attain the same effect (Fig. 9a, row 3). These simple experiments revealed 2 important facts. First, the Crp$_{P.putida}$ maintains the same RNAP-activating abilities of the *E. coli* protein; second, the *P. putida* Crp recognizes its same target DNA sequence. Yet, the affinity for the target could be lower (or expression of Crp$_{P.putida}$ lesser in the heterologous host), since overproduction of Crp$_{P.putida}$ was required to fully complement the phenotype under examination (compare rows 1 and 3 of Fig. 3). In any case, the function of the adenylate cyclase was necessary to bring about complementation, because *crp*$_{P.putida}$ failed to restore maltose metabolism in a double Δ*crp* Δ*cya*A strain of *E. coli*, even when overexpressed (Fig. 3b; see below). This is an important detail, because other members of the same Crp-FNR family of proteins have been claimed to work independently of cAMP (e.g. Clp from *Xanthomonas campestris* complements an *E. coli crp cya*A double mutant; de Crecy-Lagard *et al.*, 1990). Furthermore, Vfr of *P. aeruginosa* seems able to operate in some promoters in the absence of cAMP (Fuchs *et al.*, 2010a).

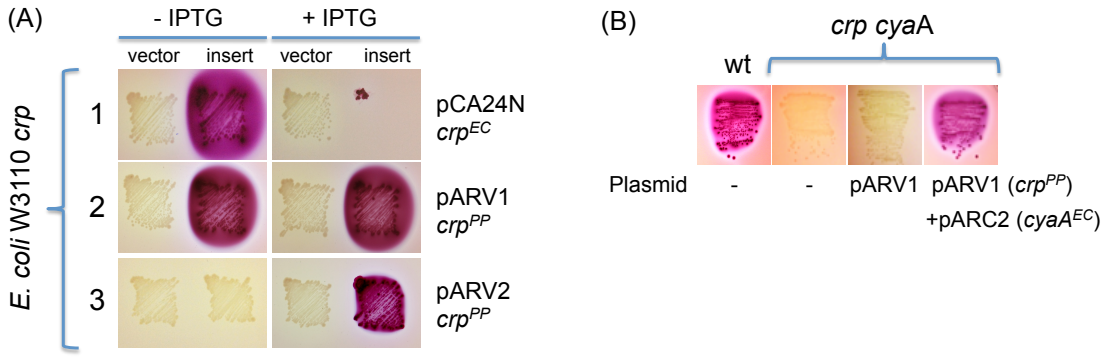


Fig. 9. Complementation analysis of *P. putida crp* and *cyaA* genes in *E. coli*. **(a)** Expression of *crp* genes in *E. coli* Δ*crp*. The host strain was transformed with plasmids bearing either the *crp* genes from *E. coli* (EC) or *P. putida* (PP) or their corresponding insert-less vectors as indicated. Each transformant was then patched onto MacConkey-maltose agar plates with or without 1 mM IPTG and incubated at 37°C. The red colour of colonies indicates maltose consumption and thus complementation of the *crp*-less phenotype. Note the toxicity exerted by $crp_{E.coli}$ when over-expressed from pCA24N*crp*. **(b)** Expression of $crp_{P.putida}$ in a double Δ*cya*A Δ*crp E. coli* mutant. High-copy number $crp_{P.putida}$-encoding plasmid pARV1 was transformed by itself in the host indicated or co-transformed with $cyaA_{E.coli}^{+}$ plasmid pARC2 in the same strain. Rescue of the Mal^{+} phenotype is observed only when the Δ*cya*A Δ*crp* strain contains the two compatible plasmids (pARV1 + pARC2).

2.3 *In vivo* functional complementation of $CyaA_{E. coli}$ by $CyaA_{P. putida}$

As mentioned above, there is only one recognizable *cya*A gene in *P. putida.* It corresponds to PP_5222 (Fig. 6B), which encodes a putative protein of 951 amino acids belonging to the class I of soluble adenylate cyclases (http://www.uniprot.org/uniprot/Q88CF9; Danchin, 1993). As it was the case with $crp_{P.putida}$, the $cyaA_{P. putida}$ gene stands alone in the chromosome, apparently encoding a mono-cistronic RNA. Thus, we implemented the same complementation assay as before to test production of cAMP borne by its cognate AC. First, the $cyaA_{P. putida}$-borne plasmid pMZC5 (Table 2) was transformed and induced in the Δ*cya*A mutant of *E. coli*. As controls we employed $cyaA_{E. coli}$ (pARC2) as well as the gene encoding the catalytic subunit of the adenylate cyclase of *Bordetella pertussis* (pARC3), which belongs to a different class of AC (Ladant *et al.*, 1992). The results of these experiments are shown in Fig. 10, rows 1-3. While the controls $cyaA_{E.coli}$ and $cyaA_{B. pertussis}$ were able to complement the lack of *cya*A in the maltose metabolism test in *E. coli*, $cyaA_{P. putida}$ failed to do so. This indicates that cAMP was not produced in *E. coli,* at least to

the levels needed to bring about complementation. On the other hand, $crp_{P.putida}$ had no effect on the lack of maltose consumption of a Δ*cya*A Δ*crp* strain of *E. coli,* even when overproduced with IPTG (Fig. 9B; see above). These results established that $Crp_{P.putida}$ does not function separately from cAMP, at least in this type of essays.

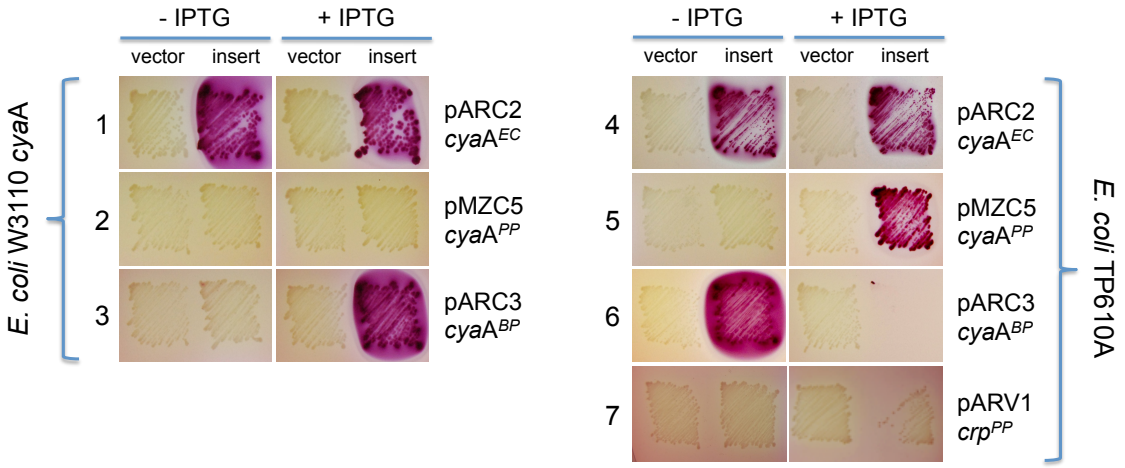


Fig. 10. Detection of cAMP production mediated by the CyaA product of *P. putida*. The Δ*cya*A strain of *E. coli* W3110 (rows 1-3) and the cAMP-hypersensitive host *E. coli* TP610A (rows 4-7) were transformed with plasmids bearing the *cya*A genes from *E. coli* (EC), *P. putida* (PP) and *B. pertussis* (BP) along with their corresponding insert-less vectors as specified. Transformants were patched onto MacConkey-maltose plates with or without 1 mM IPTG and incubated as in Fig. 9. Note that plasmid pMZC5 expressing $cyaA_{P. putida}$ failed to restore the Mal^+ phenotype in the conventional Δ*cya*A host (row 2) but it complemented the phenotype in the specialized strain TP610A (row 5). In the same conditions, the $cyaA_{B. pertussis}$ variant is toxic for the same hypersensitive host (row 6). Observe also that overproduction of $crp_{P.putida}$ (pARV1) cannot complement *E. coli* TP610A, even if overproduced (row 7), indicating that in no case $Crp_{P.putida}$ can work independently of cAMP for activation of the maltose operon.

Taken together, these data led to a paradox. On one hand, $Crp_{P.putida}$ needs cAMP to work *in vivo*. On the other hand, we were unable to detect the production of cAMP by the complementation of the *cya*A strain of *E. coli* W3110 with a plasmid expressing the orthologous gene of *P. putida* (Fig. 10; rows 1-3). One possibility is that a small molecule different from cAMP is the one that actually elicits the regulatory ability of the protein. As mentioned above, cGMP could be a candidate to this, but no recognizable guanidyl cyclases can be recognized in the genome of *P. putida,* and its residual production from side

reactions is probably insignificant (Linder, 2010). A simpler explanation could be that $Crp_{P.putida}$ has a higher affinity for cAMP in a way that allows the active cAMP-Crp complex to form with lower intracellular concentrations of the effector. Thus, the levels of the cyclic nucleotide produced by the $CyaA_{P.\ putida}$ could be significantly lower. To examine this possibility, we resorted to the use of a specialized strain of *E. coli* (named TP610A; Hedegaard & Danchin, 1985), which is hyper-sensitive to cAMP. This means that a lower intracellular concentration of cAMP is sufficient to restore the phenotypes derived from the loss of *cya*A. As before, this strain was separately transformed with expression plasmids bearing $cyaA_{E.coli}$ and $cyaA_{B.\ pertusis}$ as controls, and $cyaA_{P.\ putida}$ as test case (Fig. 10, rows 4-6). Using this experimental setup we were finally able to observe that overexpression of $cyaA_{P.\ putida}$ in this *E. coli* strain restored maltose metabolism, suggesting that cAMP was indeed produced albeit in very small amounts. On the other hand, overproduction of $crp_{P.putida}$ in the same strain had no effect (Fig. 10, row 7), thereby confirming the absolute requirement of cAMP for the protein to exert its regulatory role. An interesting sidelight of the results shown in Fig. 5 is what appears to be a strong threshold effect of intracellular cAMP concentrations. For instance, comparison of rows 3 and 6 (cells transformed with a $cyaA_{B.\ pertusis}$ expression plasmid) revealed that residual production of the protein in the non-induced cells complemented the lack of *cya*A in cAMP-hypersensitive strain *E. coli* TP610A but not in the ordinary counterpart *E. coli* W3110 *cya*A. We did not find intermediate phenotypes on MacConkey-maltose plates as judged by colony coloration, thereby pointing out that complementation of cAMP deficit was an all-on or all-off occurrence. This could explain that a lesser level of expression of $cyaA_{P.\ putida}$ in a standard Δ*cyaA E. coli* strain completely fails to complement the mutation (Fig. 10, row 2), whereas the same construct fully reinstates the phenotype in a cAMP hypersensitive strain (Fig. 10, row 5).

2.4 *In vivo* complementation with both Crp and CyaA of *P. putida* and *E. coli*

The complementation experiments described above, suggest that the adenylate cyclase of *P. putida* may have a very low activity, unable to provide enough intracellular concentrations of cAMP for activation of the indigenous Crp of *E. coli* in the W3110 *cya*A strain, but still capable to produce sufficient cyclic nucleotide to complement the same loss in the cAMP hypersensitive TP610A strain. However, it remains unclear whether the level of the cyclic nucleotide produced by its cognate CyaA is enough to activate $Crp_{P.\ putida}$. To test this

important detail, we co-transformed a double Δ*cya*A Δ*crp* mutant of *E. coli* with compatible plasmids expressing al possible combinations of *crp* and *cya*A genes from either *E. coli* or *P. putida* and examined the restoration of the Mal+ phenotype in MacConkey-maltose plates as shown in Fig. 11. The complementation assays indicated that the only combinations that produced red colonies in MacConkey plates were those expressing $CyaA_{E.\ coli}$ / $Crp_{P.\ putida}$ and $CyaA_{P.\ putida}$ / $Crp_{P.\ putida}$. This indicated that the levels of cAMP generated by $CyaA_{P.\ putida}$ were too low for activation of the protein in *E. coli,* but sufficient enough to turn on that of *P. putida*.

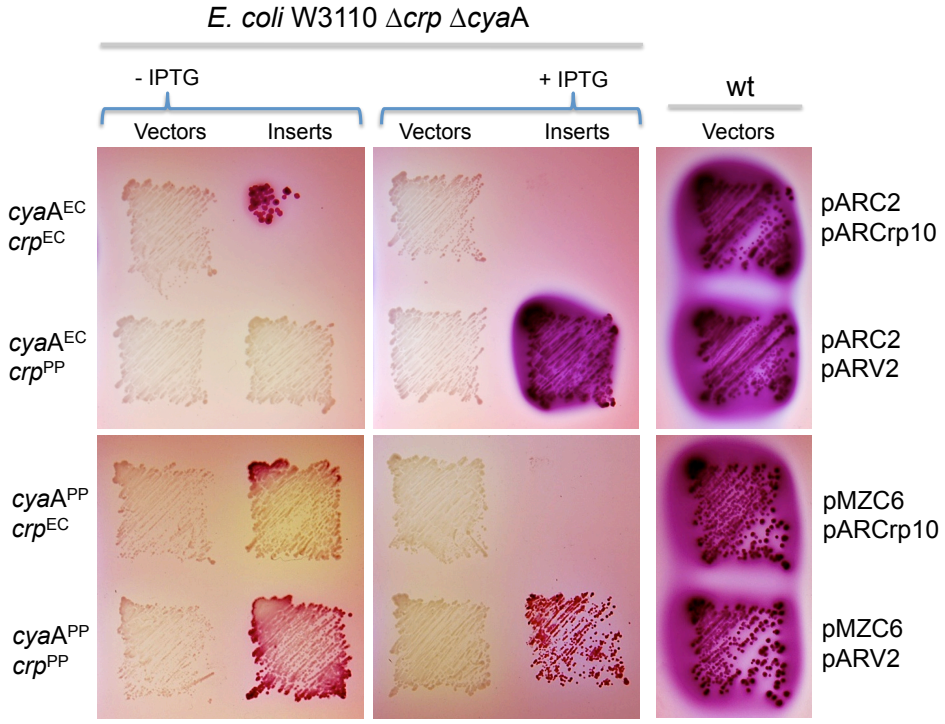


Fig. 11. Complementation of the Δ*cya*A Δ*crp* double mutant of *E. coli* with combinations of plasmids encoding *crp* and *cya*A genes of *E. coli* and *P. putida*. Each of the pairs of constructs indicated or their insert-less vectors were transformed in strain *E. coli* W3110 Δ*crp* Δ*cya*A and plated onto maltose-MacConkey medium with or without 1 mM IPTG, as shown. The right panel corresponds to wild-type *E. coli* W3110 co-transformed with empty pVLT31/pVTR-A (top) or pVLT31/pUC18Not (bottom) as controls. The red colour of the colonies (maltose consumption) indicates complementation of both *Δcya*A and *Δcrp*. Note the toxicity of overexpressing the *crp* gene of *E. coli*, which reduces or even inhibits growth of the corresponding host cells.

Taken together, the whole set of complementation experiments shown in this section indicate two important things: **[i]** $Crp_{P.\ putida}$ is an active transcriptional regulator which acts in concert with cAMP, and **[ii]** $CyaA_{P.\ putida}$ produces low levels of cAMP, insufficient to activate the Crp of *E. coli* but enough to promote the activation in $Crp_{P.putida}$, suggesting in turn that this protein could have a higher affinity for this cyclic nucleotide. These possibilities will be revisited later.

3 Concentrations of cyclic AMP in *P. putida*

3.1 Detection of cAMP by the slime mold *Dictyostelium discoideum*

On the basis of the results above we set out several approaches to detect the concentrations of cAMP in *P. putida.* To first detect the release of this compound by bacterial cells, we

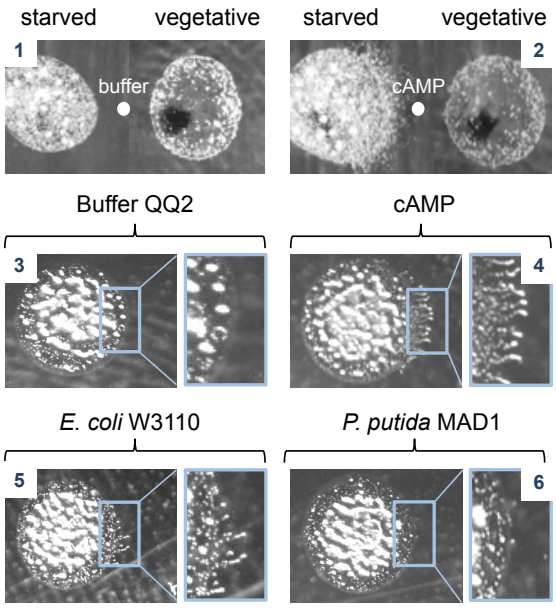


Fig. 12. *D. discoideum* cAMP chemotaxis test *in vivo.* Overnight cultures of the sensor slime mold in either nutrient-rich (allowing vegetative state) or starvation media (QQ2 buffer, see Materials and Methods), were plated in the presence of the compound specified in each case and observed with a stereoscope. Colonies previously grown in starvation media endowed *D. discoideum* with a chemotactic motion towards cAMP (panels 1-4). For the experiments, the same starved colonies were laid in the proximity of concentrated supernatants of either *E. coli* or *P. putida* cultures (panels 5 and 6). Note dendritic extensions towards the *E. coli* sample in contrast with the maintenance of the *D. discoideum* colony rim in the case of *P. putida.*

set up an *in vivo* biosensor based on the chemotaxis of *Dictyostelium discoideum* towards cAMP, which is a differentiation signal for starved cells of this slime mold at concentrations in the nM-µM range (Saran *et al.*, 2002). The corresponding experiment is shown in Fig. 12. As a control, the upper panels 1 and 2 of Fig. 12 display how the rims of starved and vegetative colonies of *D. discoideum* were affected differently by cAMP: starved cells presented a dendritic growth towards the compound that was not apparent in vegetative cells. A closer view of such colonies (Fig. 12, panels 3 and 4) allowed differentiating the presence or absence of cAMP in the neighbourhood. These conditions were used for testing the release of compounds from *E. coli* and *P. putida* cells that are able to elicit the same chemotactic response. The panels 5 and 6 of Fig. 4 indicated that this was the case for *E. coli* but not for *P. putida*, thereby confirming that the production and/or excretion of cAMP was negligible under these conditions.

3.2 Quantification of the intracellular and extracellular levels of cAMP

In order to corroborate the qualitative cAMP detection results obtained with the *D. discoideum* biosensor, we developed a sensitive HPLC-ESI-MS analytical procedure to test and quantify the levels of cAMP both in the culture supernatant and the cytoplasm of bacteria grown on minimal-glycerol medium up to the late exponential phase of growth (see Materials and Methods). Note that the quenching method was adapted for full recovery of the analyte at the moment of cell harvest. As a control of sensitivity we processed equivalent samples from wild-type and *cya*A strains of *E. coli*. It is noteworthy to point that *E. coli* wild-type cells were cultured in the conditions where they are expected to produce high amounts of cAMP (Joseph *et al.*, 1982). Conversely, the Δ*cya*A strain of *E. coli* was cultured in 0.2% (w/v) glucose due to its inability to grow on glycerol as carbon source. The results of these experiments are shown in Fig. 13. As anticipated, cAMP was found both intracellularly (~180 pmol/OD_{600}/ml) and in the external medium (~1100 pmol/OD_{600}/ml) of the cultures of wild-type *E. coli.* In contrast, the *cya*A strain gave cAMP signals below the calibration range of the method (> 43 pmol/OD_{600}/ml). Unexpectedly, we were unable to detect any cAMP signal coming from *P. putida* cells collected and processed with the same protocol. This was somewhat surprising because -as mentioned above- the complementation experiments indicated that $Crp_{P.\ putida}$ needs cAMP for its regulatory function, at least in the heterologous test system employed.

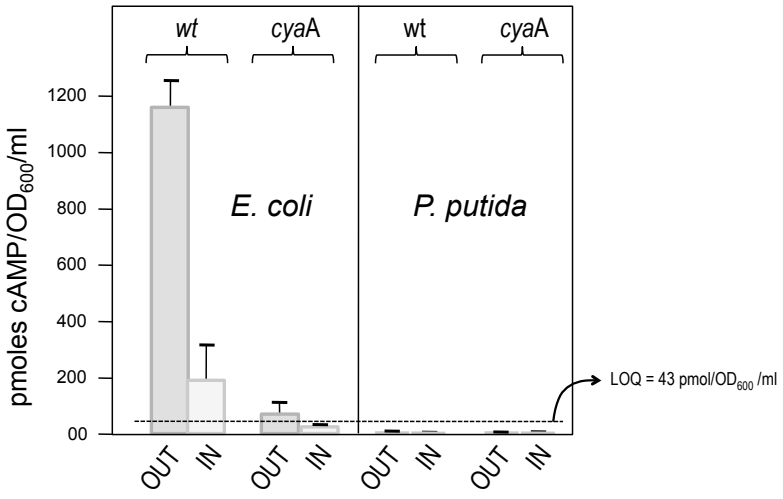


Fig. 13. Analysis of cAMP production by *E. coli* and *P. putida.* Levels of cAMP were determined in both cells (IN) and supernatants (OUT) of wild-type *E. coli* W3110 and *P. putida* MAD-1 strains along with their *cya*A counterparts by HPLC-ESI-MS as explained in Materials and Methods. The limit of quantification (LOQ) of the method employed was in the range of 43 pmol/OD_{600}/ml as indicated.

4 The *cya*A gene is transcribed, but it is not efficiently translated in *P. putida*

The complementation experiments with the *cya*$A_{P.\ putida}$ in the TP610A cAMP-hypersensitive strain of *E. coli* and the experiments for detection and quantification of cAMP in *P. putida*, confirm that the production of the cyclic nucleotide in this bacterium is lower compared to *E. coli*, at least in the conditions tested. This low intracellular cAMP production could be caused *inter alia* by low transcription/translation rates of the *cya*A gene and/or by a deficient enzymatic activity of the adenylate cyclase itself. We know by genome-wide transcriptomic analysis of *P. putida*, that the adenylate cyclase mRNA is well transcribed (Yuste *et al.*, 2006). However, the close examination of the 5' UTR of *P. putida* *cya*A mRNA in Fig. 14A reveals that the putative Shine-Dalgarno (SD) motif of this gene is suboptimal according to the GGAGG core that makes direct contact with the anti-SD sequence of the 16S rRNA (Ma *et al.*, 2002). Then, the lack of a good quality SD sequence could prevent proper formation of the translation initiation complex and therefore, the *cya*A mRNA will not be efficiently translated into the adenylate cyclase enzyme. To test this hypothesis, we constructed plasmid-borne translational (pARPcyaA1) and transcriptional (pARPcyaA2) fusions of the *cya*$A_{P.\ putida}$ promoter (P*cya*A) with *lac*Z. Initially we quantified the activity of *cya*A'-'*lac*Z translational fusion employing the classical β-galactosidase assay

described by Miller (1972). However, the values obtained were too low to be significant (i.e. <15 Miller Units, not shown). Therefore, we resorted to the ultra-sensitive Galacto-Light Plus™ commercial system. Fig. 14B shows the corresponding LacZ activity for both

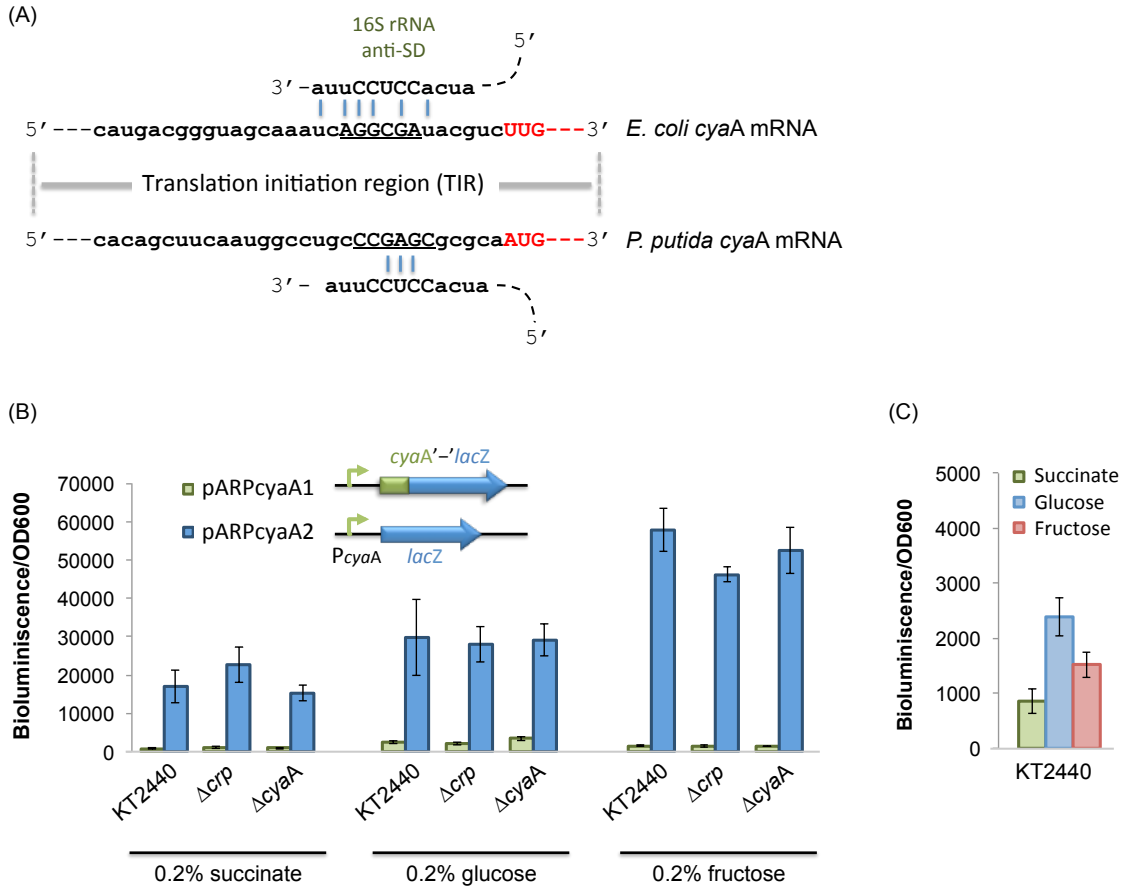


Fig. 14. Transcription and translation of the *cya*A gene of *P. putida*. **(A)** Overview of the translation initiation region of *P. putida* and *E. coli cya*A genes. The putative Shine-Dalgarno (SD) sequence of each gene is underlined and base-paired with the 3' anti-SD of the 16S rRNA. The start codons are annotated in red colour. Note the non-canonical TTG start codon of *E. coli cya*A. **(B)** β-galactosidase activity of translational (green) and transcriptional (blue) fusions of P*cya*A promoter with *lac*Z. Plasmids encoding the promoter fusions were transferred to the indicated *P. putida* strains and the enzymatic activity was measured by the Galacto-Light Plus™ method in cultures grown on succinate, glucose or fructose as carbon source. The basal level of promoterless plasmids (pSEVA225T and pSEVA225) was respectively subtracted from measured LacZ activity. The experiments were run in triplicates. **(C)** Zoom-in of the β-galactosidase activity of *P. putida* KT2440 with the translational fusion of P*cya*A (pARPcyaA1) depicted in Fig. 14B. Note the change in the scale of the y-axis.

translational and transcriptional fusions of P*cya*A, measured in the *P. putida* strains of cAMP-Crp system (KT2440, Δ*crp* and Δ*cya*A) grown on different carbon sources (succinate-glucose-fructose). The results reveal a remarkable difference amongst the transcription and the translation levels of the *cya*A$_{P. putida}$ gene, the transcriptional activity of the promoter being between 10-30 fold-change higher than the translational activity in the conditions tested. As observed, the deletion of *crp* and *cya*A did not affect the activity of the promoter, conversely to *E. coli* where the cAMP-Crp complex binds to the P2 promoter of *cya*A$_{E.coli}$ and down-regulates its expression (Mori & Aiba, 1985; Aiba, 1985). This was later confirmed by an electrophoretic mobility shift assay (EMSA) of a ~200 bp end-labelled DNA fragment comprising the upstream region of *cya*A$_{P. putida}$ gene and purified Crp$_{P. putida}$. Even at protein concentrations as high as 5 μM, Crp$_{P. putida}$ was not able to retard noticeably the electrophoretic migration of the P*cya*A DNA probe despite of the presence of cAMP (not shown).

Interestingly, the growth on fructose seems to increase slightly the transcription of the P*cya*A promoter (blue bars in Fig. 14B) compared to glucose or succinate. However, inspection of the translational fusion of this promoter in Fig. 14C reveal that the *cya*A'-'*lac*Z β-galactosidase activity is higher in glucose than in the other carbon sources. As the translational fusion demonstrate better the real amounts of CyaA protein that cells are producing (in comparison with the transcriptional fusion that reveals the mRNA levels of *cya*A), we can assume that the translational levels of CyaA$_{P. putida}$ are higher in glucose than in succinate or fructose, albeit this difference is slight.

5 The production of Crp$_{P. putida}$ is slightly increased in cells growing on glucose.

Following the same approach used for P*cya*A analysis, a plasmid-encoded *lac*Z translational fusion including the *crp* promoter (P*crp*) and the sequence of its 30 first amino acids was constructed. This vector was mobilized to *P. putida* KT2440 and its *crp*/*cya*A mutants, and the β-galactosidase activity was measured in three different carbon sources. The results depicted in Fig. 15, show that the β-galactosidase levels of the *crp*'-'*lac*Z translational fusion are high (from ~250 to ~900 Miller Units) and slightly controlled by the carbon source in which the cells are growing. Although this difference is not striking, it seems that the translational activity of *crp*'-'*lac*Z fusion is higher in cells growing on glucose than that for

the cells growing on succinate or fructose. Remarkably, expression of *cya*A'-'*lac*Z fusion is also higher when cells are growing on glucose as only carbon and energy source (Fig. 14C). Once again, the deletion of *crp* and *cya*A genes in *P. putida* did not have effect in the transcription/translation of *crp*. This last observation result was corroborated also by EMSA with purified $Crp_{P.\ putida}$ and a DNA probe containing 200 bp of the promoter region of *crp*. Even at protein concentrations as high as 5 μM, $Crp_{P.\ putida}$ failed to bind and retard the electrophoretic migration of the promoter (not shown).

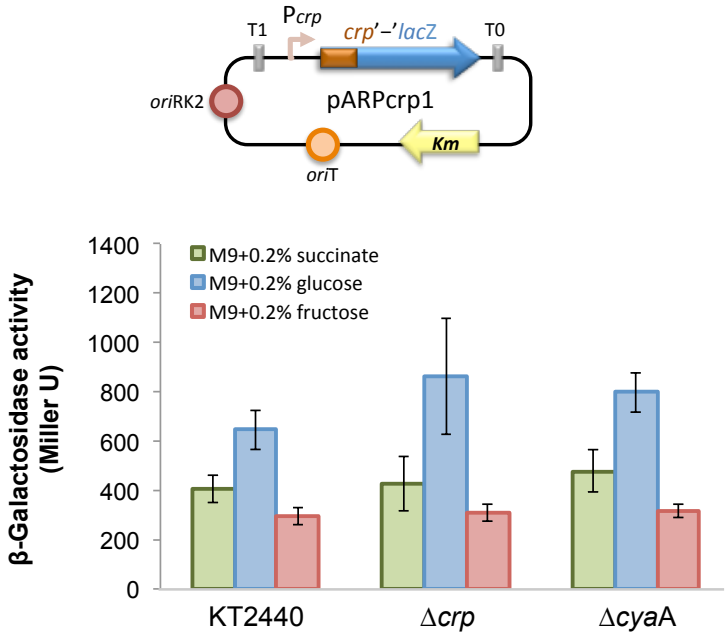


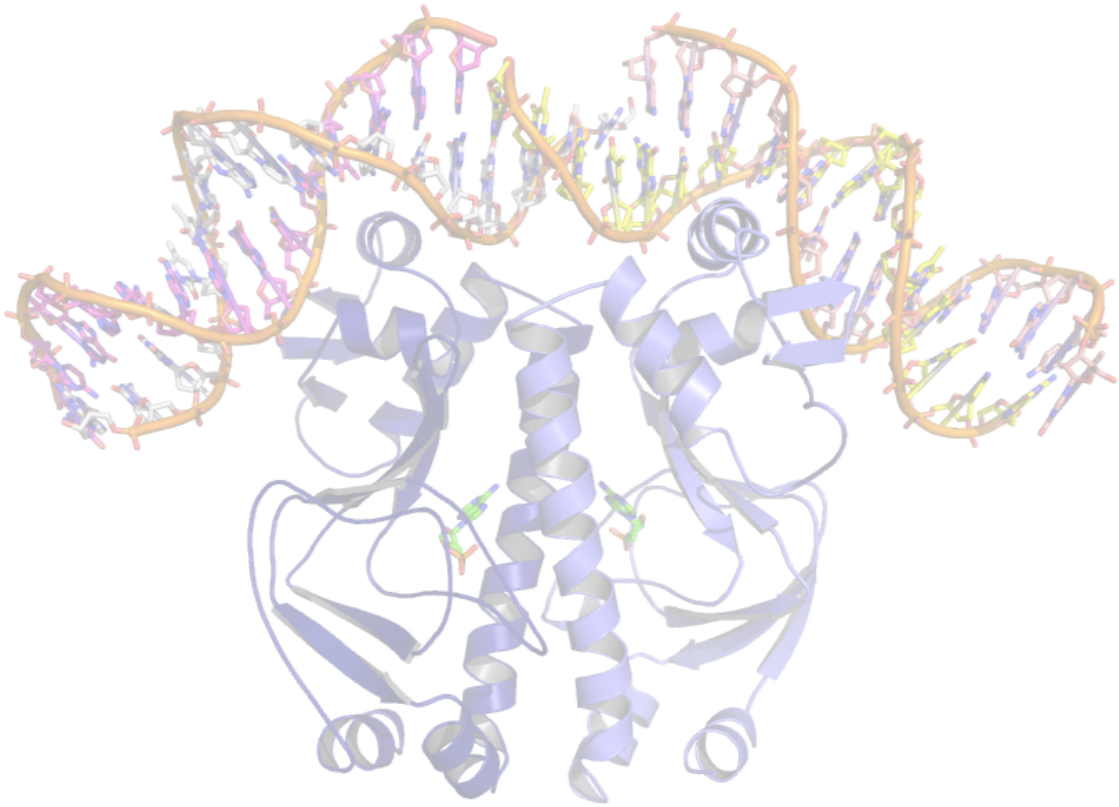
Fig. 15. β-galactosidase activity of a translational *crp'-'lacZ* gene fusion. Plasmid pARPcrp1 (upper panel) was transferred to the indicated *P. putida* strains and the enzymatic activity measured by the classical ONPG hydrolysis method (Miller, 1972) in cultures grown on succinate, glucose or fructose as carbon source. The basal activity of promoterless pSEVA225T was subtracted from measured LacZ activity. The experiments were run in triplicates.

In summary, the information collected until now demonstrates in first instance that cAMP is necessary for $Crp_{P.putida}$ activity in *E. coli*. Nevertheless, cAMP production in *P. putida* was neither detected by the *D. discoideum* biosensor or by the intracellular/extracellular quantification of the cyclic nucleotide. Moreover, complementation assays in the *E. coli* strains W3110 *cya*A and TP610A with the $cyaA_{P.\ putida}$ suggest that the synthesis of cAMP by

its cognate adenylate cyclase is very low (Fig. 10), in part because the *cya*A$_{P. putida}$ mRNA is well transcribed, but hardly translated. This state of affairs raises the hypothesis that Crp$_{P.putida}$ has a high affinity for cAMP. Hence, lower intracellular concentrations of cAMP could be enough to activate Crp and promote transcription of Crp-controlled promoters in *P. putida*. The experiments presented in the next Chapter were made to ascertain this hypothesis.

Chapter II

Biochemical characterization of *P. putida* Crp: evidence of an unusually high affinity for cAMP



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1 Purification of Crp$_{P.putida}$ as an MBP-fusion protein

Initial attempts to purify Crp as an N-terminal His-tagged fusion were completely unsuccessful due to the low amounts of soluble protein expressed in the cells, which tended to precipitate and degrade rapidly (not shown). Hence, we considered an alternative method based in the maltose binding protein of *E. coli* (MalE or MBP), which has been shown to promote the correct folding of heterologous polypeptides added to its C-terminus in a large number of cases and to maintain the resulting fusion in a soluble and active form (Bach *et al.*, 2001; Kapust & Waugh, 1999). We thus cloned the structural *crp*$_{P.putida}$ gene in the optimized vector pMAL-C2T as described in Materials and Methods. This vector is derived from pMAL-C2X (New England Biolabs), but it has been improved by exchanging the Xa protease-cleavage site (Ile–Glu–Gly–Arg) located at the boundary between the MBP domain and the protein of interest by that of thrombin (Leu–Val–Pro–Arg–Gly–Ser; Arce-Rodriguez *et al.*, 2012).

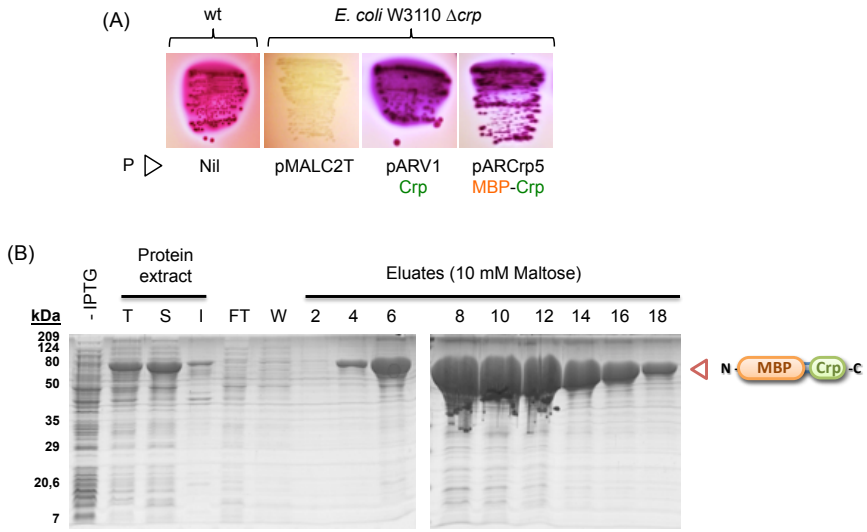


Fig. 16. Expression and purification of MBP-Crp$_{P.putida}$ in *E. coli*. **(A)** Rescue of Mal$^+$ phenotype in MacConkey-maltose agar was tested in W3110 Δ*crp* strain of *E. coli* transformed with empty pMAL-C2T and with plasmids encoding Crp (pARV1) and MBP-Crp fusion protein (pARCrp5). Utilization of maltose was observed by red-coloured colonies. **(B)** Purification steps of MBP-Crp fusion protein in an amylose-resin column. The first lane (-IPTG) correspond to the un-induced control, while the protein extracts correspond to: T: total proteins (before lysis); S: soluble fraction; I: Insoluble fraction (cell debris); FT: flow-trough (unbound proteins) and W: Washes of the resin with equilibration buffer. Eluates were obtained with equilibration buffer plus 10 mM maltose as described in the Materials and Methods.

Prior to proceed with any purification, we ensured that the resulting MBP-Crp protein was active, for which we transformed *E. coli* W3110 Δ*crp* with the plasmid pARCrp5 that encodes the fusion. As shown in Fig. 16A, such plasmid complemented the lack of *crp* in maltose MacConkey medium as well as the MBP-less counterpart. On these bases, we moved on to express and recover the MBP-Crp$_{P.\ putida}$ fusion as explained in the Materials and Methods section (Fig. 16B). The purification strategy employed, was suitable to obtain large amounts of a high-purity protein and to overcome the problem of the insolubility observed with the His-tagged version of Crp$_{P.\ putida}$.

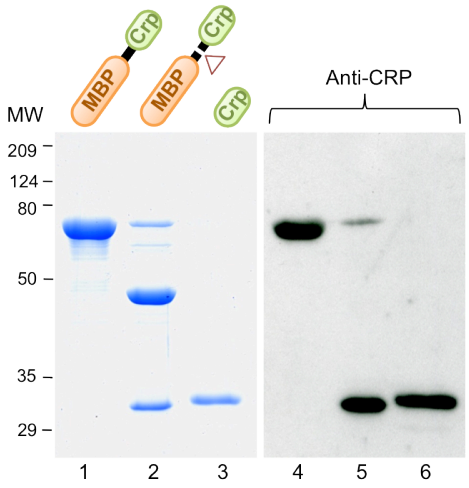


Fig. 17. Cleavage of MBP-Crp$_{P.\ putida}$ and purification of MBP-less Crp$_{P.\ putida}$. Samples from protein purification steps were analyzed by 12% denaturing PAGE stained with Coomassie blue (left panel, lanes 1-3) or subject to Western blot with rabbit anti-Crp$_{E.coli}$ serum (right panel, lanes 4-6). Lanes 1 and 4: MBP-Crp fusion protein. Lanes 2 and 5: thrombin cleavage of MBP-Crp. Lanes 3 and 6: purification of free Crp from digested MBP-Crp with phosphocellulose resin.

Next, the fusion protein was submitted to digestion with thrombin (Fig. 17, lane 2) for releasing the Crp$_{P.putida}$ moiety of the fusion. This was then captured in a phosphocellulose column by the pseudo-affinity of some DNA binding proteins for this kind of resin (Ward *et al.*, 1991) and recovered at high-salt as an apparently homogenous protein (Fig. 17, lane 3). This passover could be identified as the Crp protein by Western blot of the denaturing gel with anti-Crp$_{E.\ coli}$ rabbit serum (Fig. 1B, lanes 3-6). However, the purified Crp run in the gel with an apparent molecular mass of ~32 kDa, which diverges from the 24.571 kDa

predicted for the protein on the basis of its amino acid sequence (ProtParam tool, ExPASy server; Gasteiger *et al.*, 2005). To clarify this, we submitted the purified protein to SELDI TOF mass spectrometry, which non-only confirmed the virtual homogeneity of the Crp$_{P. putida}$ preparation, but also fixed its mass in 24.6 kDa.

2 Crp$_{P.putida}$ is a dimeric protein that undergoes a conformational change upon cAMP binding

The assays with pure Crp$_{P. putida}$ protein started by determining its oligomerization state and the potential effects of cAMP on it. To examine this issue, we resorted to analytical ultracentrifugation assays as described in the Materials and Methods section. As shown in Fig. 18, the sedimentation velocity experiments indicated that >90% of the native Crp$_{P. putida}$ has a sedimentation coefficient of 3.2 ± 0.1 S, which suggests a protein dimer to be the predominant protein form under the ultracentrifugation. On the other hand, sedimentation equilibrium assays predicted this major protein species to hold an apparent

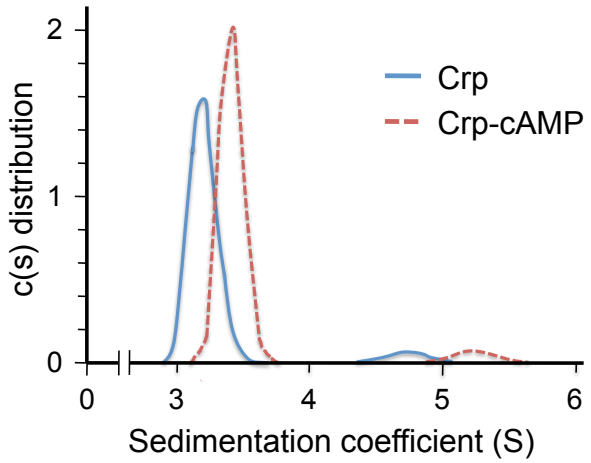


Fig. 18. Oligomerization state of *P. putida* Crp. Plotted data correspond to the sedimentation coefficient distribution obtained in sedimentation velocity experiments with purified Crp (Blue-continuous line) and Crp in complex with cAMP (Red-dotted line). A predominant fraction of Crp species (92.8%) presented a sedimentation coefficient of 3.2 ± 0.1 S. This value corresponds to a molecular mass of 51.6 kDa as confirmed by sedimentation equilibrium experiments, which is compatible with the dimeric form of the protein. Addition of cAMP to the samples increases the sedimentation coefficient value of Crp to 3.4 ± 0.1 S (93.6% of total species), corresponding to a molecular mass of 66.5 kDa.

molecular mass of 51.6 kDa. This value is virtually twice the mass predicted for the Crp monomer (24.571 kDa) and verified by mass spectrometry to be 24.6 kDa (see above). The set of data thus establishes that $Crp_{P.\ putida}$ is a stable dimer regardless of any effector. Yet, this does not rule out the possibility that binding of cAMP cause a modification in the oligomerization state of the protein, as is the case of many other transcriptional regulators like FNR (Lazazzera *et al.*, 1996), AraC (Soisson *et al.*, 1997), Lrp (Chen & Calvo, 2002) or UlaR (Garces *et al.*, 2008). To examine this possibility the same experiments were repeated but adding 1 mM cAMP to the centrifugation sample. As observed in Fig. 18, the sedimentation coefficient changed to 3.4 ± 0.1 S and the sedimentation equilibrium increased to an apparent molecular weight of 66.5 kDa, suggesting that the effector binding causes a different conformational change in the cAMP-$Crp_{P.\ putida}$ complex with respect to the effector-less protein.

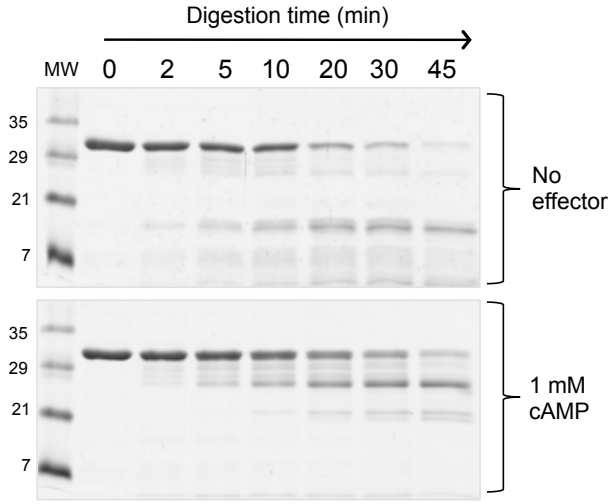


Fig. 19. Partial proteolysis of $Crp_{P.\ putida}$. The purified protein was subject to digestion with trypsin in the absence (top panel) and presence (bottom panel) of 1.0 mM cAMP. Reactions were stopped at the times indicated and the samples were loaded in a 15% denaturing PAGE stained with Coomassie blue. First lane corresponds to the molecular weight marker.

The analytical ultracentrifugation data before suggest that the hydrodynamic ratio of Crp dimer may somehow change upon cAMP binding. To ascertain this hypothesis, we implemented a simple test for detecting large conformational transitions based on partial

proteolysis of the purified protein with trypsin in the presence and absence of cAMP (Krakow & Pastan, 1973). Fig. 19 shows a Coomassie blue-stained gel displaying the peptides resulting from digestion of protein samples incubated or not with cAMP. Although the pattern of tryptic peptides is difficult to interpret in structural terms because of the anomalous migration of Crp discussed before, its seems that a large protein domain becomes released by the protease upon exposure to cAMP, a feature compatible with the conformational change exposed above. Furthermore, inspection of the gels of Fig. 19 revealed no share of any of the proteolysis intermediates between the preparation devoid of cAMP and containing the effector. This strengthens the notion that the cAMP-unbound and cAMP-bound conformations of the factor represent two different forms of the protein.

3 Crp$_{P.putida}$ displays an extraordinary high affinity for cAMP

The results obtained from complementation assays and the detection of cAMP in *P. putida* cells disclosed in Chapter I, raised the hypothesis of a higher affinity Crp$_{P.putida}$, which could be activated by the low intracellular levels of cyclic nucleotide produced by its native CyaA. Thus, to quantify the interplay between Crp$_{P.\ putida}$ and cAMP we set out to employ Isothermal Titration Calorimetry (ITC), one of the most reliable and accurate techniques to calculate a wide range of molecular interaction parameters (Krell, 2008). Since this procedure requires high protein concentrations, long handlings of $\geq$ 2.5 h and the sample undergoes considerable changes in temperature, we first chose the MBP-Crp$_{P.putida}$ hybrid protein (see above) instead of the native-sized factor for such ITC assays. As mentioned above, the MBP domain does not change the characteristics of the protein *in vivo* while it helps its solubility both *in vivo* and *in vitro* (Fig. 16).

Fig. 20A shows a typical titration curve of MBP-Crp$_{P.\ putida}$ with cAMP, which yields the thermodynamic parameters listed in Table 4. The fitting of the data with the *One Binding Site* model gave rise to a very satisfactory solution, indicative of a single event of molecular interaction. The results obtained reveal that binding is driven by favourable changes in the enthalpy (ΔH) and entropy ($T\Delta S$) of the system, the process resulting in an exothermic reaction. In contrast, non-cyclic AMP (Fig. 4B, upper profile) produced no interaction signals with the factor, even when tested at very high concentrations. Moreover, inspection

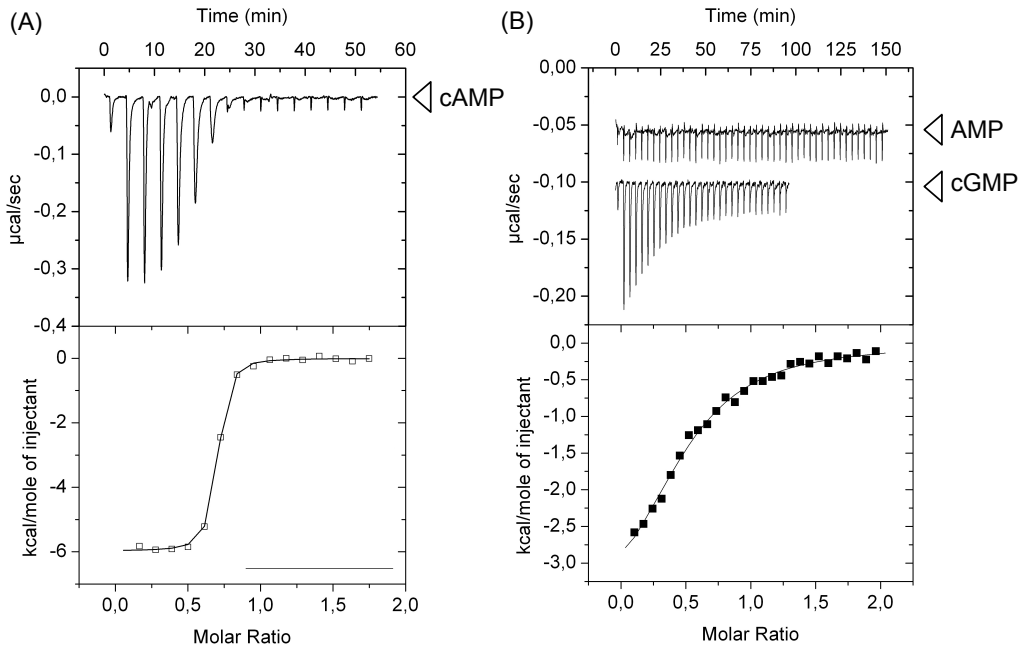


Fig. 20. Isothermal titration microcalorimetry of MBP-Crp*P. putida* interacting with putative effector molecules. Purified MBP-Crp fusion protein was titrated with dilutions of (**A**) cAMP (**B**) AMP, cGMP, as indicated. The upper panels plot the raw data obtained from titration of 10 µM of protein with aliquots of effector molecules at 350 µM (for further details see Materials and Methods). The lower panels represent the integrated and dilution-corrected curves fitted from raw data with the *One Binding Site* model of the MicroCal version of ORIGIN software. Thermodynamic parameters obtained from curves are summarized in Table 4.

of data in Table 4 and Fig. 20A revealed also an outstanding high affinity of MBP-Crp*P. putida* for cAMP with a dissociation constant K_D = 22.5 ± 2.8 nM, much lower than the figures for the homologous protein of *E. coli* (Gorshkova *et al.*, 1995). This result reveals the biological reason of the low levels of cAMP found in this bacterium, i.e. as Crp*P. putida* displays its *ultratight* avidity for the cAMP, the intracellular concentration of the cyclic nucleotide necessary to activate this protein must be significantly lower.

As stated above, the S128T substitution in the *E. coli* Crp protein is known to make the mutant responsive also to cGMP (see Chapter I). In *P. putida* the equivalent residue in this position is the T133, bringing about the possibility that Crp*P.putida* uses cGMP as an effector as well (see Fig. 8). Hence, a second set of ITC experiments were done with cGMP instead of cAMP. The experiment of Fig. 20B indicates that, similarly to the titration with cAMP, cGMP binds to MBP-Crp*P. putida* through an exothermic reaction driven by favourable

Table 4. Thermodynamic parameters obtained from titration of either MBP-Crp*P. putida* fusion protein or apo-Crp*P. putida* with putative effectors.

Ligand	Stoichiometry	K_A (M^{-1})	K_D	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
MBP-Crp*P. putida*						
cAMP	0.65 ± 0.01	(4.4 ± 0.5) x 10^7	22.5 ± 2.8 nM	-5.97 ± 0.03	4.47 ± 0.1	-10.44 ± 0.1
cGMP	0.49 ± 0.02	(3.9 ± 0.4) x 10^5	2.6 ± 0.3 µM	-4.16 ± 0.27	3.46 ± 0.3	-7.62 ± 0.06
AMP	*nd*[a]					
apo-Crp*P. putida*						
cAMP	0.498 ± 0.002	(2.2 ± 0.2) x 10^7	45.0 ± 3.4 nM	-4.89 ± 0.03	5.11 ± 0.05	-10.0 ± 0.1
cGMP	0.327 ± 0.084	(1.7 ± 0.2) x 10^5	5.7 ± 0.7 µM	-15.24 ± 4.3	-8.09 ± 4.3	-7.15 ± 0.07

[a]. No binding was observed for non-cyclic AMP

changes in the enthalpy and entropy (Table 4). However the affinity of the cGMP for MBP-Crp*P.putida* is ~100 fold lower than that of cAMP under the same conditions (dissociation constant K_D = 2.6 ± 0.3 µM). Despite the less defined shape of the ITC curve, the experimental results could be satisfactorily fit to the *One Binding Site* model (Table 4).

4 Crp*P.putida* binds one cAMP molecule per protein dimer

Regardless of the high quality of results obtained from MBP-Crp*P.putida* titrations with the data fitted in the *One Binding Site* model, the stoichiometry of the binding reaction remains ambiguous. For example, the stoichiometry of the cAMP-Crp*P. putida* complex deduced from Fig. 20A is approximately 0.65, which is close to 0.5 (it means 1 molecule of cAMP per dimer of Crp) and occurred in a single binding event, describing a monophasic curve. This is an important outcome to take into account, because all the Crp-like proteins described so far bind two or more cyclic nucleotides per protein dimer (Weber & Steitz, 1987; Passner & Steitz, 1997; Stapleton *et al.*, 2010; Tao *et al.*, 2010; Cordes *et al.*, 2011). However, the 0.65 *n* stoichiometry value obtained for cAMP binding to MBP-Crp*P.putida* is

not precise enough to determine truthfully the number of cyclic nucleotide molecules that activates this protein. Therefore, we decided to modify the purification protocol and overexpress the MBP-Crp$_{P.putida}$ fusion protein in *E. coli* W3110 Δ*crp* Δ*cya*A grown on M9-glucose medium, in order to obtain a Crp$_{P.putida}$ devoid of any source of cAMP (see Materials and Methods). The reason of this new approach is because, despite the extensive washing and dialysis steps during purification, it is likely that MBP-Crp$_{P.putida}$ is copurified with traces of cAMP due to its high affinity for the cyclic nucleotide. Also, we optimized the ITC reaction buffer conditions by increasing concentrations of KCl to 250 mM and by adding 1 mM DTT, in order to sustain a reducing milieu for the 6 cysteines of the protein. These minor changes allowed stabilizing the Crp$_{P.putida}$ and keeping it in the soluble form. Together, these set of modifications allowed us conducting the ITC experiments again with *bona fide* cAMP-less Crp of *P. putida* (apo-Crp$_{P.putida}$) cleaved from MBP.

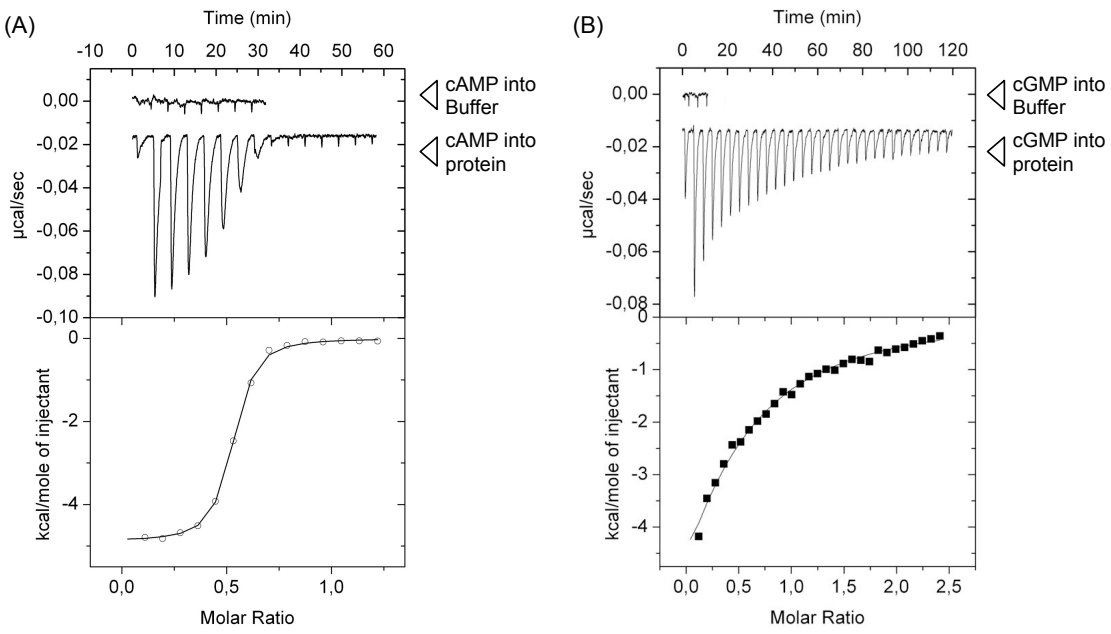


Fig. 21. Isothermal titration microcalorimetry of apo-Crp$_{P. putida}$ interacting with **(A)** cAMP and **(B)** cGMP. The upper panels plot the raw data obtained from titrations with the cyclic nucleotides. The first titration in these panels exhibits the injection of the effector molecule into buffer without protein. The lower panels represent the integrated and dilution-corrected curves fitted from raw data with the *One Binding Site* model of the MicroCal version of ORIGIN software. Note that the titrations with cAMP were conducted in a buffer containing 250 mM KCl, against the 150 mM used for same experiments with cGMP. Thermodynamic parameters obtained from curves are summarized in Table 4.

The Fig 21A shows the titration of MBP-less apo-Crp$_{P.putida}$ with cAMP. The hyperbolic curve traced from plotting the titration data is virtually identical to the one observed for MBP-Crp$_{P.putida}$, the binding being driven by favourable changes in the enthalpy (ΔH) and entropy ($T\Delta S$) of the system (Table 4). Likely, the interaction between both molecules yields once again a tight binding for the cyclic nucleotide (K_D = 45 ± 3 nM). In spite of these similarities, the affinity for the cyclic nucleotide in the apo-protein is somewhat reduced as compared to the initial data set using the fusion protein (Table 4). However, this minor discrepancy is likely to be caused by the increased in the ionic strength to 250 mM KCl and not by the absence of the MBP tag. Moreover, integration of data with the *One Binding Site* model results in a very satisfactory fit, which describes only one binding event and confirms without any doubt the stoichiometry of 1 molecule of cAMP per dimer of Crp$_{P.putida}$. This reflects a scenario in which only one of the two cAMP binging sites available in the protein becomes occupied.

Similarly, the titration of apo-Crp$_{P.putida}$ with cGMP plotted in Fig. 21B was very similar to the interaction of the cyclic nucleotide for the MBP fusion protein (Fig. 20B). Nevertheless, in this case the binding of cGMP is driven by negative entropy changes (Table 4). This unfavourable ΔS value can have different reasons and is not necessarily caused by an ordering of water molecules at the complex interface. Another important negative contribution to the entropy change is the loss of flexibility of the ligands during the binding process (Jelesarov & Bosshard, 1999). Thus, the cGMP-Crp$_{P. putida}$ complex could occur in a way where the interaction process does not alter the conformational architecture of the binding sites and therefore, the entropy of the system is negative. Once again, the affinity of the cGMP for apo-Crp$_{P.putida}$ is ~100 fold lower than the one observed for cAMP with the same protein, which confirms the results obtained with the MBP-Crp$_{P.putida}$ fusion protein. Furthermore, as was the case with cAMP interaction for apo-Crp$_{P.putida}$, there is just one binding event for cGMP-Crp$_{P.putida}$ complex. Despite the less defined shape of the ITC plot, the *One Binding Site* model fitting of the data yields again a binding stoichiometry closer to 0.5 (0.327 ± 0.084, Table 4), which is consistent with the model proposed that only one molecule of the cyclic nucleotide (cAMP or cGMP) is interacting with the Crp$_{P.putida}$ dimeric protein.

5 Crp$_{P.\ putida}$ activates the transcription machinery of *P. putida* in a class I Crp-dependent promoter

Since the specific target sequences of Crp$_{P.\ putida}$ in the native host genome are still uncertain but its gene complements the metabolic phenotypes of a Δ*crp* mutant of *E. coli* (Fig. 9), we assumed that the *P. putida* protein should bind and activate typical class I Crp-dependent promoters such as P*lac* (Busby & Ebright, 1999). Thus, a 330 bp DNA fragment containing the native P*lac* promoter was PCR amplified and either end-labelled for gel retardation (EMSA) assays or recloned in the pJCD01 specialized vector for creating a supercoiled template for *in vitro* transcription (see Materials and Methods for details).

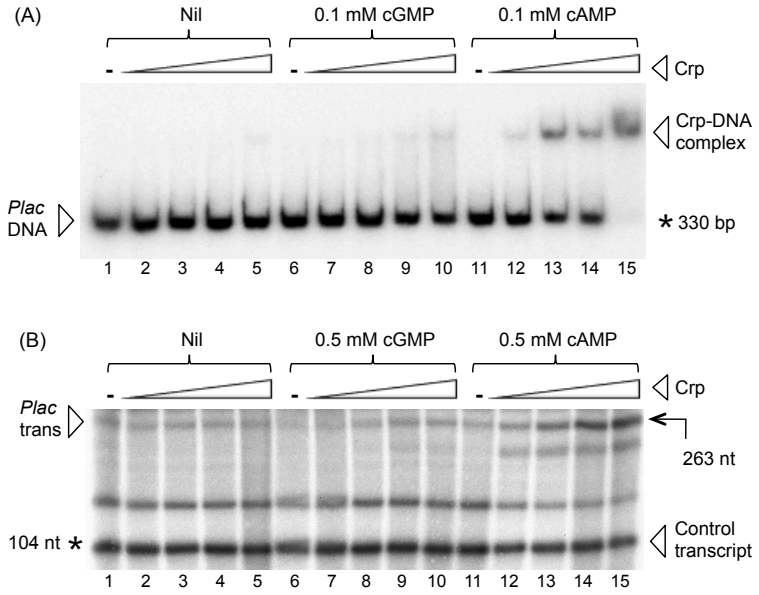


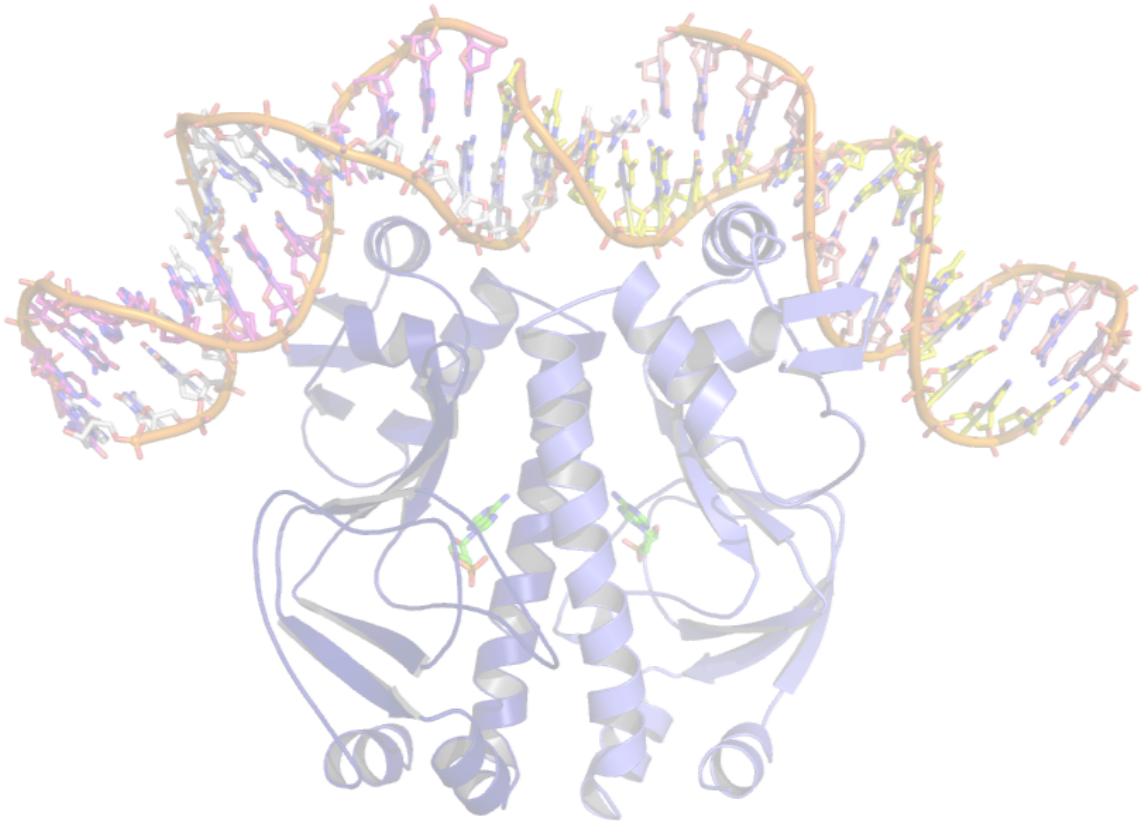
Fig. 22. DNA recognition and transcription activation of the P*lac* promoter mediated by Crp$_{P.\ putida}$. (**A**) Electrophoretic mobility shift assay (EMSA) of a 330 bp fragment containing the P*lac* promoter with increasing concentrations of Crp and its effectors. Binding reactions contained 0.5 ng of end-labeled P*lac* probe, 0.1 mM of cyclic nucleotides (cAMP and cGMP) and increasing concentrations of Crp$_{P.\ putida}$ (lanes 2, 7 and 12: 10 mM; lanes 3, 8 and 13: 50 nM; lanes 4, 9 and 14: 100 nM; lanes 5, 10 and 15: 250 nM. (**B**) *In vitro* transcription of P*lac* promoter with purified Crp and σ^{70} RNAP of *P. putida*. P*lac* were PCR cloned into plasmid pJCD01 and the reactions were carried out with increasing concentrations of Crp$_{P.\ putida}$ in the presence and absence of either 0.5 mM of cGMP or 0.5 mM cAMP. Crp concentrations were as follows: lanes 2, 7 and 12: 10 nM; lanes 3, 8 and 13: 20 nM; lanes 4, 9, and 14: 50 nM; lanes 5, 10 and 15: 100 nM. The specific 263 nt P*lac* transcript and the 104 nt internal control from plasmid vector pJCD01 are indicated at both sides of the film.

Fig. 22 shows the corresponding experiments, in which the activities of Crp$_{P.\ putida}$ were tested using an excess of either cAMP or cGMP as components of the reaction mixtures. Inspection of the EMSA data of Fig. 22A indicated that cAMP was necessary for binding Crp$_{P.\ putida}$ to the DNA fragment containing the *lac* promoter, the gross K_D of the DNA-protein interaction being in the range of 50 nM. In contrast, the effector-less protein failed to retard noticeably the labeled probe even at concentrations as high as ~ 0.3 μM (Fig. 22A, lane 5). On the other hand, cGMP appeared to increase minimally such a residual binding but only at the highest protein concentration –far above any biological sense. Since the levels of cGMP employed in the assay (100 μM) were nearly 20 times higher than the K_D calculated with ITC using the apo-Crp$_{P.\ putida}$ (see above), we can assume that the same protein form that binds cGMP fails to bind DNA, at least the Crp recognition sequence employed present in the labeled DNA probe.

Next, we set up the *in vitro* transcription experiment of Fig. 22B to corroborate whether results obtained in the EMSA experiments are correlated with the transcriptional activation of the P*lac* promoter. Hence, the same DNA fragment (cloned in an specialized vector) was incubated with RNA polymerase and Crp$_{P.\ putida}$ with identical effectors. Note that the enzyme employed was purified from *P. putida* (see Materials and Methods) and therefore the transcription assay recreates the conditions that RNAP and Crp experience in its native host. Inspection of Fig. 5B tells us that a weak *lac* transcript which is produced by the RNAP alone (lane 1) is not increased by either addition of the effector-less Crp$_{P.\ putida}$ (lanes 2-5) or by its complex with cGMP (lanes 7-10). Yet, the same band intensifies in the samples with the cAMP-Crp$_{P.\ putida}$ complex, as would be expected of Class-I Crp-dependent promoters like P*lac* (Busby & Ebright, 1999). This set of data also confirms that only cAMP binding triggers the Crp$_{P.\ putida}$ protein for DNA binding and transcription activation, whereas the interaction with cGMP leads instead to a non-productive protein form.

Chapter III

Detection of genes directly or indirectly regulated by the components of the cAMP-Crp system in *P. putida*



1 RNA-seq transcriptome analysis of *crp* and *cya*A mutants of *P. putida*

The previous Chapters of the Thesis demonstrated, from phenotypically and mechanistically points of view, the mechanism by which Crp interacts with the allosteric effector cAMP in order to activate the transcription of some Crp-dependent promoters in *E. coli*. However, the data said nothing on the functional scope ruled by the *crp* and *cya*A gene products in the native *P. putida*. The development of *omics* technologies (transcriptomics, proteomics, metabolomics, etc.) have emerged in recent years as powerful tools to address this important piece of information. Thus, we decided to perform a genome-scale transcriptome analysis of the Δ*crp* and Δ*cya*A mutants of *P. putida* compared with the wild-type KT2440 strain. For this purpose, we implemented the newly developed high-throughput sequencing using the Illumina RNA-seq methodology, instead of classical microarray technologies. RNA-seq allows the sequencing of millions of short DNA fragments in a single run, which are derived from reverse transcription of bacterial RNA (previously fragmented and cleaned up from rRNA, see Materials and Methods). These fragments are then aligned with the entire *P. putida* genome and mapped with annotated genes. The information obtained by sequencing data enables the identification of differentially expressed genes, while allowing for additional analysis, such as detection of low-expressed genes, novel transcripts and identification of ncRNAs (Marioni *et al.*, 2008). Following the procedures detailed in the Materials and Methods section, we analysed the transcriptome of *P. putida* strains KT2440, Δ*crp* and Δ*cya*A previously grown until mid-exponential phase in M9 medium supplemented with 0.2% glucose. We decided to use glucose as the only carbon source because the translational fusions of *lac*Z gene with both *crp* and *cya*A promoters showed slightly higher β-galactosidase activity in cells growing in this carbon source, compared to others as succinate or fructose (see Fig. 14C and Fig. 15).

The differential expression of genes in the *P. putida* Δ*crp* strain, compared with wild-type KT2440, is shown in Fig 23. We found many genes that were either upregulated or downregulated in the Δ*crp* mutant. Thus, it is plausible that $Crp_{P.\ putida}$ could act as a dual transcriptional regulator, either activating or repressing the expression of several transcriptional units. However, we also find that there are more genes overexpressed (65 genes) in the Δ*crp* strain than the ones whose expression is downregulated in the same mutant (only 43 genes), compared to wild-type strain. This transcriptome analysis

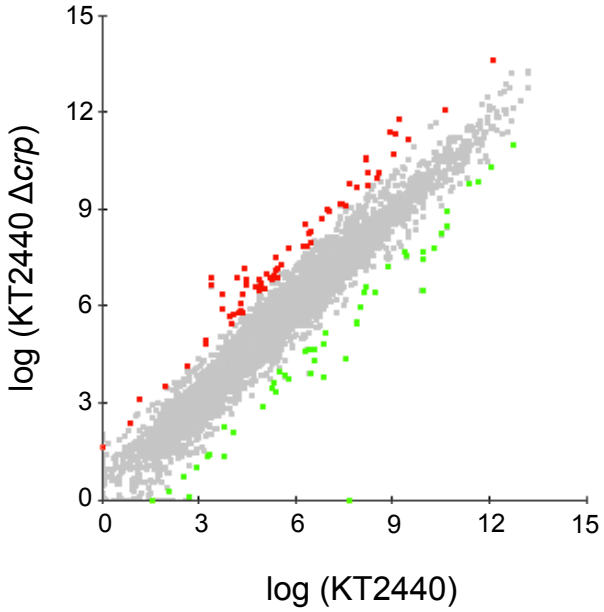


Fig. 23. Differential expression pattern of genes in the Δ*crp* mutant of *P. putida* compared with reference KT2440 wild-type strain. The RNA of cells cultured in M9-glucose medium was isolated and analysed by Illumina high-throughput sequencing as explained in Materials and Methods section. Upregulated genes: Red; Downregulated genes: Green. Detail of the genes differentially expressed could be found in Table 5 and 6 of the annexes section.

is both preeliminary and limited and it does not certify whether the differences in gene expression are the effect of direct or indirect interactions between Crp and those genes. Yet, the abundance of upregulated versus the downregulated genes in the *P. putida* Δ*crp* mutant stated above suggests that Crp could behave mainly as a transcriptional repressor. Tables 5 and 6 in the annexes describe respectively the upregulated and downregulated genes in the Δ*crp* mutant of *P. putida*, together with the predicted function and subcellular localization of their protein products. Unfortunately, many of these genes codify hypothetical proteins or proteins with a hypothetical function. Still, it is noteworthy that only few genes related with carbon source metabolism or transport were found to be regulated by $Crp_{P.\ putida}$. These genes include an acetyl-CoA synthetase (*acs*A), a benzoate transporter (PP_1820) and the gene *glc*G, which form part of the *glc* operon for glycolate utilization in *E. coli* (Pellicer *et al.*, 1996). In contrast, many of the proteins produced by genes regulated by $Crp_{P.\ putida}$ (overexpressed and also repressed) are related with cell membrane functions and therefore, are predicted to localize in the cell membrane. These proteins include some outer membrane receptors, protein secretion systems, transporters and pili subunits (see Tables 5

and 6 in the annexes for details). This results agree with the observation made by Milanesio (2007) that cAMP-Crp system controls mainly cell surface-related functions.

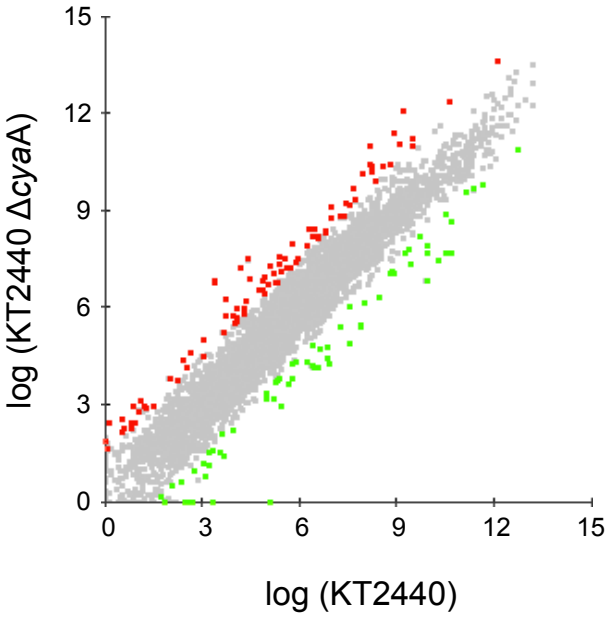


Fig. 24. Differential expression pattern of genes in the Δ*cya*A mutant of *P. putida* compared with reference KT2440 wild-type strain. The RNA of cells cultured in M9-glucose medium was isolated and analysed by Illumina high-throughput sequencing as explained in Materials and Methods section. Upregulated genes: Red; Downregulated genes: Green. Detail of the genes differentially expressed could be found in Tables 7 and 8 of the annexes section.

On the other hand, Fig. 24 shows the differential expression of genes in the Δ*cya*A strain of *P. putida*, compared with wild-type KT2440. A detailed list of the upregulated and downregulated genes plotted in Fig. 24 can be found respectively in Tables 7 and 8 of the annexes. As in the case of Δ*crp* mutant of *P. putida*, in Δ*cya*A strain we found more upregulated genes (81 genes) than downregulated ones (57 genes), with respect to wild-type strain. Remarkably, virtually none of them matches with previously annotated genes related to the metabolism of carbon sources. This ruled out, once again, the general assumption that cAMP is a key signal for metabolization of none-preferred carbon sources in bacteria as it does in *E. coli.*

There were a larger number of genes that are controlled (either directly or indirectly) by the *cya*A gene product, compared to genes under the regulation of $Crp_{P.\ putida}$. It is noteworthy

that many genes that were up- or downregulated in the Δ*crp* mutant were also regulated in the same fashion in the Δ*cya*A strain. This convergence is showed in the Venn diagrams of Fig. 25A (upregulated genes) and Fig. 25B (downregulated genes). We found some interesting transcriptional units (genes and operons) shared by both mutants. For example, the *arc*DAIC operon for the metabolism of arginine via the arginine deiminase pathway is upregulated in both the Δ*crp* and the Δ*cyaA* strains. Another small operon overexpressed in both mutants is conformed by the locus PP_0353 and PP_0354. The first gene encodes for a putative exonuclease, while the second gene is annotated as a CBS (cystathionine-β-synthase) domain protein. The later gene is very relevant not only because it is the upregulated gene that exhibits the maximum fold change in both Δ*crp* and Δ*cya*A strains, but also because it includes a predicted cyclic nucleotide binding domain. Additionally, some overexpressed operons include proteins related to cell membrane functions. This is the case of the ABC protein secretion efflux system (included in the operon formed by locus PP_0803 to PP_0806), the *cfa* cyclopropane-fatty-acyl-phospholipid synthase (included in the operon formed by locus PP_2733 to 2738) or the biosynthesis of lipopolysaccharides carried out by WecB protein. Furthermore, components of the type I pili (encoded in the operon formed by locus PP_2357-2363) were found to be upregulated in both mutants. Finally, we also found the genes encoding for cytochrome C oxidase subunits and the assembly protein (operon formed by locus PP_0103 to PP_0111) to be upregulated in both mutants of *P. putida*.

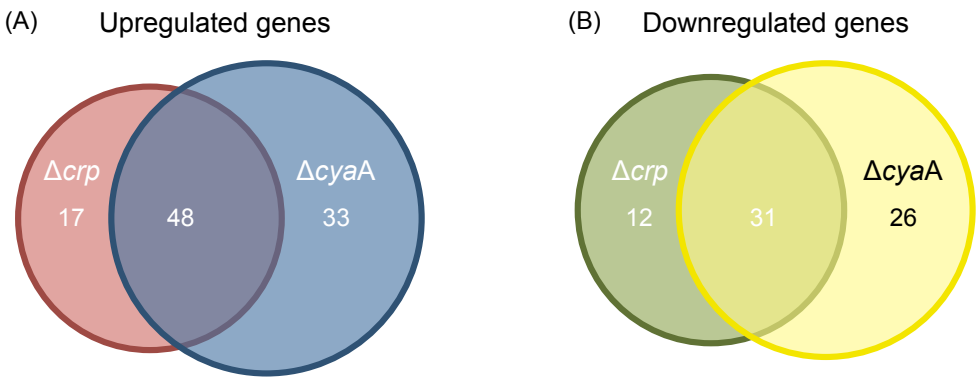


Fig. 25. Venn diagrams illustrating the genes that are co-regulated by Crp and CyaA in *P. putida*. Figure shows the up-regulated **(A)** or down-regulated **(B)** genes shared in common in the Δ*crp* and Δ*cya*A strains respective to wild-type KT2440.

We also detected an important set of genes that were downregulated in the Δ*crp* and the Δ*cyaA* strains. Interestingly, we found that some of these genes were involved in the protection of the bacterial cell against environmental stresses. These include the genes coding for the major chaperones in the microbial cell: GroES, GroEL and DnaK. Additionally, we found another two heat-shock proteins (*ibp*A and *htp*G) and a putative chaperone-associated ATPase (PP_3095). Thus, cAMP-Crp system could be involved in the activation of the cell response to heat or environmental stress. We observed three operons that include several genes encoding outer membrane receptors, like the outer membrane protein OprG, the outer membrane copper receptor OprC and the putative ferric siderophore receptor PP_3330. Finally, another genes downregulated in both Δ*crp* and Δ*cya*A mutant strains of *P. putida* include a flavin mononucleotide (FMN) reductase (*ssu*E), the translation initiation factor IF1 (*inf*A) and the ribosomal protein L31 (*rpm*E).

Despite most of the genes that are upregulated or downregulated in the Δ*crp* mutant, are also affected in the Δ*cya*A strain in a similar fashion, there are another set of genes that were only affected in one mutant or in the other, respective to the wild-type strain. This is especially true in the case of the Δ*cya*A mutant of *P. putida*. Thus, it is tempting to suggest that there could be another partners and factors involved in the cAMP signalling pathway in *P. putida*. This fascinating issue is currently under examination.

V. Discussion

1 The cAMP-Crp system of *Pseudomonas putida*

P. putida bears in its chromosome an authentic cAMP-Crp gene regulation system similar to that of *E. coli*, encoding at least one copy of its three main components: the adenylate cyclase (*cya*A, PP_5222), the cAMP-receptor protein (*crp*, PP_0424) and the cAMP phosphodiesterase (*cpd*A, PP_4917). The next question to address was whether this system also conserves the biological function and the gene regulation mechanism as its enterobacterial counterpart, where Crp is at the top of the hierarchy in the control of carbon metabolism (Kolb *et al.*, 1993; Ishihama, 2010; Gama-Castro *et al.*, 2011; Shimada *et al.*, 2011). If this feature is also conserved in *P. putida*, then the metabolism of carbon sources in this bacterium would be tightly controlled by cAMP-Crp complex. However, the growth of *crp* and *cya*A deficient strains of *P. putida* on succinate, glucose and fructose (each processed through different metabolic pathways) is mostly indistinguishable from the growth of wild-type KT2440. Recent works are consistent with this result and show that deletion of *crp* and *cya*A genes in *P. putida* hardly alter the global metabolic features in this bacterium (Daniels *et al.*, 2010; Milanesio *et al.*, 2011). Moreover, the implication of *P. putida* cAMP-Crp could be related with cell surface functions, as resistance to toxic compounds, cell motility and biofilm formation (Milanesio, 2007). On this background, we implemented a simple but very informative assay in which we complement the loss of *crp* and *cya*A genes in *E. coli.* The complementation of *E. coli* W3110 *crp* with the plasmid encoding $crp_{P.putida}$, not only demonstrates that $Crp_{P.putida}$ is a functional transcriptional regulator, but also reveals three important pieces of information: first, $Crp_{P.putida}$ can recognize the *E. coli* Crp binding sequence of the maltose-consumption promoters regulated by this TF. As shown in Fig. 8, the HTH DNA binding domain of $Crp_{P.putida}$ is conserved with regards to its *E. coli* counterpart. This high level of homology, together with the results of maltose complementation in *E. coli* explained before, suggest that $Crp_{P.putida}$ recognizes similar (but perhaps non-identical) DNA target sequences in its native host. Second, $Crp_{P.putida}$ is able to contact the $RNAP_{E. coli}$ and to promote the transcription of Crp-dependent promoters (Fig. 9). Third, Fig. 8 also shows the conservation in $Crp_{P.putida}$ of the residues encompassing these three activating regions responsible for the interaction with the RNA polymerase in *E. coli*. Just two of these activating regions (AR1 and AR2) seem to occur naturally in the $Crp_{E. coli}$. The third non-native AR3 becomes artificially activated when the Lys52 of the protein surface is substituted by a residue with negative charge.

Interestingly, *P. putida* Crp has a negatively charged Glu in this position. This simple substitution could in turn activate the AR3 and therefore it can trigger the activation of class II Crp-dependent promoters by all AR1, AR2 and AR3. Finally, the lack of complementation in the *E. coli* Δ*crp* Δ*cya*A double mutant only with the plasmid bearing the $crp_{P.\ putida}$ suggests that the encoded Crp is functional when the adenylate cyclase is also present (Fig. 9B).

2 *P. putida* CyaA produces very low levels of cAMP

The complementation of *E. coli* W3110 Δ*cya*A suggests that $cyaA_{P.\ putida}$ expression gives rise to only small amounts of cAMP, just enough to complement the mutation in the hypersensitive TP610A strain. Moreover, this complementation was only achieved in this strain when the $cyaA_{P.\ putida}$ was overexpressed with high concentrations of IPTG (1 mM). This low concentration of the cyclic nucleotide was further confirmed qualitatively by the *D. discoideum* biosensor and quantitatively by HPLC-ESI-MS. While the intracellular and extracellular concentrations of cAMP detected in *E. coli* cultured on glycerol were within the range reported by others (Wayne & Rosen, 1974; Joseph *et al.*, 1982), the levels of this metabolite in *P. putida* were below the quantification limit of the method employed (> 43 pmol/OD_{600}/ml). Former analysis of cyclic AMP concentration in related proteobacteria by Phillips & Mulfinger (1981), reported mean intracellular levels of this metabolite in the range of 0.19-0.30 pmol/OD_{600}/ml for *P. aeruginosa* (the levels vary slightly depending on the carbon source in which cells are growing), 0.016 pmol/OD_{600}/ml for *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*) and 0.105 pmol/OD_{600}/ml for *Cupriavidus oxalaticus* (formerly *Pseudomonas oxalaticus*). Moreover, the same authors report intracellular cAMP levels in *P. putida* ranging values of 0.038, 0.050 and 0.029 pmol/OD_{600}/ml in cells growing on glucose, histidine and succinate, respectively. Although these results were obtained with a different methodology to that used in this work (cAMP radio-immunoassay), they also reflect the very low concentrations of cyclic AMP produced by *P. putida* and *P. aeruginosa* regardless of the carbon source where the cells are cultured. But, what is the reason for such a low cAMP production in this bacterium? The simplest and more logic explanation is to attribute a lower catalytic activity to the corresponding cyclase. In *E. coli*, the only known mechanism to influence CyaA performance (and thus

intracellular cAMP levels) involves direct contacts of the enzyme with an uncharacterized regulatory factor of the cell cytoplasm and with the phosphorylated forms of EIIA-type enzymes of the phosphoenolpyruvate-dependent carbohydrate transport system (Park *et al.*, 2006). Whether this mechanism is conserved in *P. putida* is unknown, but certainly, fructose is the only sugar that is transported through PTS in this microorganism (Velazquez *et al.*, 2004; Chavarria *et al.*, 2012). It would be surprising that the Crp–cAMP system were linked only to this sugar. Instead, it is likely that CyaA$_{P. putida}$ activity is controlled by other, so far unknown physiological or environmental signals. Another explanation to the low cAMP levels could be the action of one or more phosphodiesterases encoded in the *P. putida* genome. Here, we report a gene in this bacterium (PP_4917) encoding a protein with high homology to the *P. aeruginosa* and *E. coli* CpdA phosphodiesterases (Fig. 6D). Although there is not sufficient information about the functional duties of this protein, the high homology and the preservation of the 13 residues absolutely conserved amongst all the class III phosphodiesterases suggest that PP_4917 encodes a genuine 3'-5' cyclic AMP phosphodiesterase.

But besides the cAMP turnover played by the activity of the adenylate cyclase and the phosphodiesterase(s) enzymes, another factor that contributes to the low concentration of the cyclic nucleotide in *P. putida* is the expression of the corresponding cyclase itself. The transcriptional and translational fusions of the *cya*A$_{P. putida}$ promoter show that while this gene is efficiently transcribed in a cell population, its translation is extremely low. This feature however is not alien to *cya*A$_{P. putida}$, as several important regulatory genes as *mal*T (main regulator of maltose regulon), *nag*C (repressor/activator of the *nag* regulon, responsible for the metabolism of the amino sugars N-acetylglucosamine and glucosamine) and *trp*R (repressor of the tryptophan regulon) are also inefficiently translated in *E. coli* (Ozbudak *et al.*, 2002). Moreover, the *cya*A genes of a variety of Gram-negative bacteria (including enterobacteria) have also a slow translational rate, compared with higher levels of transcription (Trotot *et al.*, 1996). Low translational rates in these important set of genes seems to be a mechanism to regulate the phenotypic variation generated by the noise (coefficient of variation) in the protein expression. This noise is often harmful, because it disrupts the fine-tuning regulation of cell signalling pathways (Ozbudak *et al.*, 2002). Thus, a gene with low transcription, but high translation rates, produces a so-called *translational burst*, in which the gene with the higher translational efficiency *vs.* the lower mRNA

abundance displays protein bursts that are variable and infrequent, resulting in strong stochastic fluctuations in protein concentration. Conversely, a gene with high transcription and low translation rates produces burst that are small and frequent, causing only week fluctuations in protein concentration and producing a smaller phenotypic variation in the cell population (Kaern *et al.*, 2005; Ozbudak *et al.*, 2002). Therefore, the inefficient translation of *cya*A along Gram-negative bacteria (including *P. putida*) is perhaps conserved because it supresses the harmful fluctuations in CyaA and thereby in the cAMP levels that could have pleiotropic effects for the cell.

3 CyaA and Crp levels are slightly increased in cells growing on glucose and are not autoregulated by cAMP-Crp

Interestingly, the translational activity of *cya*A'-'*lac*Z fusion results to be slightly higher in *P. putida* cells growing on glucose than in cells cultured in fructose or succinate (Fig. 14C). A similar result was observed for the translational levels of *crp*'-'*lac*Z fusion of Fig. 15, in which the LacZ activity was slightly increased in glucose compared with the other carbon sources employed. If this assumption is true, then the cAMP-Crp system in *P. putida* could be active when the cells are growing on glucose, acting opposite to the same mechanism in *E. coli,* where glucose is a repressor of the adenylate cyclase activity. This speculation is not meaningless because in *P. putida* glucose is not transported by the classical PTS system as in *E. coli,* but it is metabolized via the Entner–Doudoroff pathway (del Castillo *et al.*, 2007; Chavarria *et al.*, 2012). However, the metabolic or environmental signals that control the activity of the $CyaA_{P.\ putida}$ are not known yet and they must be closely studied to ascertain the influence of glucose in the expression of the cAMP-Crp system in this bacterium.

The expression of both P*cya*A and P*crp* was not influenced by autoregulation of the cAMP-Crp complex, because deletion of *cya*A or *crp* hardly had any effect over the β-galactosidase activity of the translational or transcriptional fusions. This fact contrasts with *E. coli,* where the *cya*A expression seems to be negatively regulated by cAMP-Crp complex (Mori & Aiba, 1985; Aiba, 1985). Also expression of $crp_{E.coli}$ is controlled by a dual autoregulation mechanism in which cAMP-Crp exerts both positive and negative regulation depending on the cAMP concentration, by binding to two different Crp sites in the *crp* promoter (Ishizuka *et al.*, 1994). Moreover, *P. aeruginosa* Vfr positively regulates its own expression

in a cAMP dependent mechanism (Fuchs *et al.*, 2010a). These dissimilar regulatory modes of *crp* and *cya*A genes, due to the distinct promoter architecture amongst each bacterial species, reflects the different regulatory roles by which the cAMP-Crp system were evolutionary selected in these bacteria.

4 $Crp_{P.putida}$ is a dimeric protein that undergoes a conformational change upon cAMP binding

Analytical ultracentrifugation experiments with purified $Crp_{P.putida}$ were employed to assess its oligomerization state. This important issue cannot be predicted from protein homologies only, as often the same enzyme or regulator may adopt diverse oligomeric forms with different properties, kinetic or otherwise in different species (Chavarria *et al.*, 2011). The results show in Fig. 18 clearly established that this protein form dimers in solution regardless of any effector. Remarkably, the presence of cAMP causes an increase in the apparent size of the dimeric protein from of 51.6 kDa to 66.5 kDa, evidencing for the first time the molecular interaction between purified $Crp_{P.putida}$ and cAMP *in vitro*. Since the molecular weight contribution of the cAMP to the complex is not enough to explain this notable increment (the molecular weight of cAMP is ~329 Da) and the corresponding parameters are incompatible with formation of a higher-order oligomer (which should be at least ~75 kDa for a trimer and ~100 kDa for a tetramer), the data suggest that the hydrodynamic shape of the dimer change upon cAMP binding to produce a different protein form. This conformational change was further confirmed by partial proteolysis of $Crp_{P.putida}$ and cAMP- $Crp_{P.putida}$ complex with trypsin. Recently, Popovych *et al.* (2009) deciphered the structure of *E. coli* Crp in the absence of cAMP and proposed a mechanism of allosteric activation mediated by cAMP in which the binding of the cyclic nucleotide to the protein induces a coil-to-helix transition that extends the C-helix (responsible for Crp dimerization). This in turn places the F-helix (responsible for DNA recognition) in the correct orientation for DNA binding (Fig. 2). It is likely that a similar allosteric mechanism is occurring in the $Crp_{P.putida}$ upon cAMP binding, bringing about a strong conformational shift in the protein surface that changes the accessible sites for trypsin proteolysis as well and thus generates the different peptide digestion pattern observed in Fig. 19.

5 Crp$_{P.putida}$ displays an extraordinary high affinity for cAMP

Throughout the Chapter I of this work, we have addressed the low levels of cAMP in *P. putida* and its implication in the cAMP-Crp mechanism of gene regulation. We argued that Crp could have evolved a higher sensitivity for cAMP as a consequence of the much lower intracellular levels of the cyclic nucleotide produced by its weak adenylate cyclase. This hypothesis was finally demonstrated by the ITC experiments with MBP-Crp$_{P.putida}$ (Fig. 20A) and also with the apo-Crp$_{P.putida}$ (Fig. 21A). This binding is driven by an exothermic reaction with favourable (negative value) changes in the enthalpy (ΔH) and also favourable (positive value) entropy ($T\Delta S$) of the system. These negative enthalpy variations with cAMP (Table 4) can be explained by formation of non-covalent bonds between the cAMP molecule and the corresponding interaction pocket in Crp$_{P.\ putida}$. On the other hand, positive entropy is systematically associated to expulsion of protein-bound water to the aqueous surrounding induced by ligand binding (Krell *et al.*, 2007). Moreover, we found the affinity for cAMP as *ultratight* (K_D = 22.5 ± 2.8 nM) and also much stronger than the average affinity of effector molecules for transcriptional regulators. Compared with reference *E. coli*'s Crp protein, we found that the window of responsiveness of Crp$_{P.putida}$ to cAMP levels might vary more than 100-fold. This sensitivity is also higher than the observed for the Vfr of *P. aeruginosa* (~45-fold; Cordes *et al.*, 2011)) and surprisingly much higher than Crp of *Mycobacterium tuberculosis* (>2,000-fold; Stapleton *et al.*, 2010)). Even more, it is noteworthy that the cAMP–Crp system of *M. tuberculosis* seems to have evolved exactly in the opposite direction than *P. putida* (Stapleton *et al.*, 2010). In this case, the protein has decreased its affinity for cAMP to compensate for much higher physiological levels of the cyclic nucleotide produced by its numerous adenylate cyclases.

The strength of the binding for the related cyclic guanosine monophosphate was also demonstrated by ITC experiments (Fig. 20B and Fig. 21B). Although this binding has near 100-times lower affinity for Crp$_{P.putida}$ than the one reported for the cAMP, it is comparable with the values observed for cAMP binding to the Crp counterpart of *E. coli*, and thus it is still considerable in biological terms (Table 4). Yet, the corresponding affinities make a difference, because the strengths of the interaction of Crp$_{P.\ putida}$ with either cAMP or cGMP in *P. putida* differ two orders of magnitude. This stands in contrast with the smaller distance between the affinities of either effector in the *E. coli* scenario (Lin & Lee, 2002).

6 A negative cooperativity model of cyclic AMP binding explains the 1:1 Crp$_{P.\ putida}$-dimer:cAMP stoichiometry

As explained in the Results section, we purified Crp$_{P.\ putida}$ in a cAMP-free environment in order to determine exactly the stoichiometry of the reaction between the protein and its effectors. As observed in Fig. 21A, titration of apo-Crp$_{P.\ putida}$ with cAMP was satisfactorily fitted with the *One Binding Site* model, yielding very similar thermodynamic parameters than the interaction of MBP-Crp$_{P.\ putida}$ fusion protein with the same effector. Moreover, we observed only one single event of binding (the curve shape is monophasic) with a stoichiometry of ~0.5, which would indicate a binding of a single molecule of ligand to a dimer. Surprisingly, the stoichiometry of cAMP–Crp binding in *E. coli* has not been to this day entirely solved. While the mainstream view adopts 1:1 effector/monomer ratios (Weber & Steitz, 1987), other structural studies with cAMP–Crp co-crystals suggest instead a 2:1 proportion (Passner & Steitz, 1997). On the other hand, ITC experiments by Gorshkova and colleagues (1995) display heat changes compatible with an overall stoichiometry of two cAMP per protein monomer. Finally, Lin and Lee (2002) reported a biphasic curve by fluorescence titration and a clearly three-phasic ITC curve, indicative of two high-affinity sites and one low-affinity site. This indicates features of the protein-effector interplay in Crp$_{P.\ putida}$ that differ notably in respect of what is known about the Crp protein of *E. coli*. The fact that the ITC data show only one interaction event, reflects a scenario in which only one of the two cAMP binding sites available in the protein dimer becomes occupied. How this may happen? It is plausible that binding of one effector molecule to one of the subunits of the dimer decreases the affinity of the corresponding site in the other subunit (Milligan & Koshland, 1993) in a *negative cooperativity* effect. Hence, the bindings of the first cAMP molecule to one of the Crp$_{P.\ putida}$ monomers induce a conformational change (discussed above) that greatly lowers the affinity in the binding pocket of the remaining monomer. Counter-intuitive as it may look, this mechanism has been observed in other cases, such as the binding of aspartate to the Tar receptor of *Salmonella typhimurium* (Milligan & Koshland, 1993) or that of the flavonoid phloretin to the TtgR regulator of *Pseudomonas* (Teran *et al.*, 2006). Similarly, the results of Fig. 21A most likely reflect that cAMP:Crp stoichiometry of the *P. putida* protein is 1 ligand : 1 dimer molecule, different from the variety of scenarios in *E. coli* where Crp dimer binds two or more cAMP molecules. In addition, the experiments were repeated with cGMP as effector of apo-Crp$_{P.}$

putida (Fig. 21B). Once again, the experimental results could be satisfactorily fit to a *One Binding Site* model with a binding stoichiometry closer to 0.5 than 1 (0.327 ± 0.084, Table 4), which would indicate a binding of a single molecule of ligand to a dimer. cGMP is thus likely to interact with Crp*P. putida* through the same negative cooperativity model discussed for cAMP.

7 Crp*P. putida* activates the transcription machinery of *P. putida* and *E. coli*

After documenting the binding of cAMP and cGMP to Crp*P. putida* we next examined whether this binding result triggers the two principal biological functions attributed to this factor in bacteria, i.e. DNA binding and transcriptional activation. Fig. 22A shows that Crp*P. putida* recognizes and binds the 22 bp Crp site of the *lac* promoter (3'-TAATGTGAGTTAGCTCACTCAT-5'). Furthermore, consumption of maltose was also complemented when the *E. coli crp* mutant was transformed with the plasmid encoding the *crpP. putida*, as it is shown in Fig. 9. Thus, it is likely that Crp*P. putida* recognizes a sequence similar to the consensus of *E. coli*. On the other hand, *in vitro* transcription assays suggest that this protein do not just bind to the Crp site of the *lac* promoter, but it also bends the DNA and interact with the AR1 of the *P. putida* RNA polymerase in a similar fashion that occurs in *E. coli*. It is remarkable that DNA recognition and transcription activation occurs only when cAMP is present in the reaction, while addition of cGMP or absence of any effector does not promote binding and transcription reactions (see Fig. 22B). This set of data confirms [i] that only cAMP poises the Crp*P. putida* protein for binding DNA and activating transcription and [ii] that interaction with cGMP leads instead to a non-productive protein form.

8 RNA-seq transcriptome analysis of Δ*crp* and Δ*cya*A mutants of *P. putida*

The differential expression of genes in the Δ*crp* and Δ*cya*A mutants of *P. putida* was examined by transcriptome analysis using high-throughput sequencing of the bacterial RNA (RNA-seq). This newly developed technology presents some advantages to the classical DNA microarrays, such as the detection of novel transcripts and ncRNAs that are transcribed from the intergenic regions. Also, this methodology allows to detect low-

expressed genes and to identify alternative promoters within genes and operons (Marioni *et al.*, 2008; Wilhelm & Landry, 2009). It is important to mention that a deep analysis of the putative novel transcripts and the ncRNAs obtained by RNA-seq of several *P. putida* strains are currently under extensive examination, and therefore, these results are not presented in this work. However, we decided to present in this Thesis a limited analysis of the differential expression pattern in the Δ*crp* and Δ*cya*A strains compared to wild-type KT2440 to assess a preliminary landscape of the functional scope of Crp and CyaA proteins in *P. putida*.

The first notable feature that emerged from our transcriptome analysis is the absence of metabolic genes related with consumption of carbon sources in the Δ*crp* and Δ*cya*A mutants of *P. putida.* This stands in contrast with the situation in *E. coli,* where the shortfall of Crp results in appreciable gross changes in many operons encoding transporters and enzymes for carbon source utilization (Gosset *et al.*, 2004). Conversely, our results suggest that the cAMP-Crp complex could be involved in the regulation of genes whose function is associated with the cell membrane. We found that deletion of *crp* and *cya*A genes downregulated expression of several operons that codify for proteins involved in the response to environmental stress like GroEL, GroES, DnaK and several heat shock proteins. These mutants also repress the expression of outer membrane receptors and putative membrane associated proteins. On the contrary, Δ*crp* and Δ*cya*A strains of *P. putida* overexpress several operons involved in the biosynthesis of cell membrane components, protein secretion systems, type I pili and also genes related to the assembly of cytochrome C components. Thus, we hypothesize that cAMP-Crp$_{P.\ putida}$ complex could regulate the response to environmental stress through the activation of cell chaperones and heat-shock proteins. Simultaneously, cAMP-Crp$_{P.\ putida}$ regulates the expression of membrane-related components to shape the cell envelope in response to the harmful condition. Additionally, we found that cAMP-Crp$_{P.\ putida}$ also controls one operon for the metabolism of arginine, and that mutation of *cya*A upregulates the expression of an aldolase involved in the biosynthesis of phenylalanine, tyrosine and tryptophan. This is consistent with the observation made by Daniels *et al.* (2010) and Milanesio *et al.* (2011) in which deletion of *crp* or *cya*A impaired the use of various amino acids and dipeptides as nitrogen sources.

It was surprising that despite most of the genes altered in the *crp* mutant were also affected in the *cya*A-deleted strain, some other genes were not shared by both mutants. In *P. aeruginosa* it has been shown that cAMP was not required for Vfr binding to the *las*R promoter *in vitro* or for activation of the *las*R promoter activity *in vivo* (Fuchs *et al.*, 2010a). Given the similarity between $Crp_{P.\ putida}$ and Vfr, it is possible that the *P. putida* regulatory protein also works in a cAMP-independent manner for the regulation of some promoters. Also, in *P. aeruginosa* it was recently discovered an additional cAMP-binding protein (CbpA) that seems to be more structurally similar to eukaryotic protein kinase A (PKA) regulatory subunits than to Crp-FNR family of transcriptional regulators. This protein is localized at the cell pole upon cAMP interaction, and this localization seems to be critical to its function (Endoh & Engel, 2009). Although we did not find homologues of CbpA in the genome of *P. putida* KT2440 (results not shown), this does not rule out the possibility to find another kind of proteins whose activity is controlled by cAMP. One strong candidate is the CBS-domain protein encoded by the locus PP_0354. We found in our transcriptome analysis that this protein not only was highly overexpressed in the Δ*crp* and Δ*cya*A mutants of *P. putida*, but it also has a cAMP-binding domain similar to CBD of Crp that could be modulating its function. These possibilities, which are beyond the scope of this Thesis, will be the subject of future studies.

9 cAMP-Crp system of gene regulation: an example of *Regulatory exaptation*

One of the corollaries of the work described above is that -despite similar mechanistic performance- the cAMP-Crp regulatory device of *P. putida* seems to be mostly alien to the biological (metabolic) functions that shape its predominant duty in *E. coli.* It is perfectly possible that some metabolic promoters of *P. putida* are still under a degree of control by Crp, but either there is a physiological backup to them, or the effect is not strong enough to be detected through the growth phenotypes described above and reported by Milanesio *et al.* (2011) and Daniels *et al.* (2010). We conclude then that the very same cAMP-Crp component (along with one or more phosphodiesterases) has been recruited in different bacteria to control dissimilar sets of biological functions. It is difficult to trace the evolutionary history of such a device, but cAMP seems to be produced in virtually all biological systems (Botsford & Harman, 1992) and thus appears to be a versatile signal-

carrier molecule between regulatory modules. Whether the metabolic control functions of the system appeared earlier or later, we argue that the cAMP-Crp arrangement constitutes an obvious occurrence of evolutionary exaptation at a molecular level. This concept, which originated in Darwinian Zoology (Gould & Vrba, 1982) refers to the process by which a fixed feature that was originally selected to perform a given function, may later execute another quite unlike role, i.e. is co-opted for its current function. The archetypical example of exaptation is the evolutionary history of feathers, which were probably selected first for thermal insulation, and only later they were co-opted for flight. The key angle here is that functional re-assignment happens with little or no concurrent structural modification. In this way, one specific trait that has evolved under one set of conditions can be co-opted to serve a different function under a second set of circumstances. In our case, it is most likely that an ancestral form of the cAMP-Crp system was selected for controlling expression of one or more distinct functions, but was then co-opted in subsequent hosts to regulate entirely different functional ensembles. Even different strains of the same species can select the same transcriptional factor for controlling dissimilar sets of functions. For instance, a *crp* mutant of the solvent-tolerant *P. putida* strain DOT-T1E was unable to employ ammonium salts as sole N source (Daniels *et al.,* 2010) in contrast with the soil and rhizosphere-thriving *P. putida* KT2440 counterpart described in this work. That habitat-dependent exaptation happens in transcriptional factors argues in favour of the idea that regulatory control systems can evolve in a fashion that is somewhat independent of the functions that they regulate (de Lorenzo & Perez-Martin, 1996). Perhaps this is not true for all types of proteins, but the basic function of the cAMP-Crp system seems to enjoy a considerable level of orthogonality (i.e. context-independence; Silva-Rocha & de Lorenzo, 2008) that accounts for its regulatory flexibility in diverse bacteria.

VI. Conclusions

The data presented in this Thesis grant the following conclusions:

1. The cAMP-Crp system of gene regulation in *P. putida* is composed by at least three components: one cAMP-receptor protein (Crp), one adenylate cyclase (CyaA) and one (and maybe more) cAMP phosphodiesterase (CpdA).
2. $Crp_{P.\ putida}$ is a functional transcriptional regulator that is allosterically regulated by cAMP to complement the maltose consumption phenotype in *E. coli*.
3. The intracellular and extracellular levels of cAMP produced by the adenylate cyclase of *P. putida* are very low compared to enterobacterial organisms. The low levels of this second messenger stem from the fact that the adenylate cyclase gene is transcribed but it is not efficiently translated.
4. $Crp_{P.\ putida}$ is a dimeric protein that undergoes a conformational change upon cAMP binding. This binding is proposed to place the protein in the correct orientation to facilitate DNA recognition and binding.
5. $Crp_{P.\ putida}$ has an *ultratight* avidity for cAMP, which likely determines the window of functionality exerted by the protein in this bacterium. This hypersensitivity for cAMP helps to explain the low levels of this metabolite produced in *P. putida.*
6. $Crp_{P.\ putida}$ binds cAMP with 1:1 Crp dimer:cAMP stoichiometry. This is explained because binding of cAMP to the first protein monomer induces a conformational change that greatly reduces the affinity in the *binding pocket* of the remaining monomer
7. The cAMP-$Crp_{P.\ putida}$ complex binds and activates the transcription of the class I Crp-dependent *lac* promoter.
8. The regulatory tasks of the cAMP-Crp system in *P. putida* are related with the cellular response to environmental stress and the expression of cell membrane components to shape the cell envelope in response to such conditions. Additionally, this system could regulate the metabolism of amino acids as carbon and nitrogen sources.

El trabajo descrito en esta Tesis permite proponer las siguientes conclusiones:

1. En *P. putida,* el sistema de regulación cAMP-Crp se compone de al menos tres componentes: una proteína de unión a cAMP (Crp), una adenilato ciclasa (CyaA) y una (y posiblemente más de una) fosfodiesterasa de cAMP (CpdA)
2. La proteína $Crp_{P.\ putida}$ es un regulador transcripcional que se activa mediante regulación alostérica por el cAMP para complementar el fenotipo de metabolismo de maltosa en *E. coli*.
3. La concentración intracelular y extracelular de cAMP producida por la adenilato ciclasa de *P. putida* es muy baja en comparación con las enterobacterias. Estos niveles tan bajos son en parte debido a que el gene que codifica la adenilato ciclasa en este organismo es transcrito eficientemente, pero su traducción es muy baja.
4. La proteína $Crp_{P.\ putida}$ forma dímeros cuando se encuentra en solución. Estos dímeros sufren un cambio conformacional cuando interaccionan con el cAMP, lo que posiblemente posiciona la proteína en la orientación correcta para reconocer e interaccionar con las correspondientes secuencias de ADN.
5. La proteína $Crp_{P.\ putida}$ tiene una afinidad extraordinaria por el cAMP, que a su vez determina en gran parte sus funciones en *P. putida*. Esta hipersensibilidad por el cAMP explica por qué las concentración de cAMP en esta bacteria son tan bajas.
6. La proteína $Crp_{P.\ putida}$ se une al cAMP con una estequiometría de 1 dímero de Crp: 1 molécula cAMP. Esto se explica mediante un modelo de cooperatividad negativa en el cual la unión del cAMP en uno de los monómeros induce un cambio conformacional que reduce en gran medida la afinidad por el cAMP en el bolsillo de unión de ésta molécula en el segundo monómero.
7. El complejo formado por cAMP-$Crp_{P.\ putida}$ es capaz de unir y activar la transcripción del promotor *lac* (tipo I para Crp).
8. La función reguladora del sistema cAMP-Crp de *P. putida* está relacionada con la respuesta celular al estrés ambiental y con la expresión de componentes de la membrana que modifican el envoltorio bacteriano en esas condiciones. Adicionalmente, este sistema puede regular el metabolismo de ciertos aminoácidos como fuentes de carbono y nitrógeno.

VII. References

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VIII. Annexes

Table 5. List of upregulated genes in *P. putida* KT2440 Δ*crp vs*. wild-type KT2440 strain in M9-glucose cultures.

Locus	Gene	Fold Change	FDR	Product	Subcellular localization
PP_0354	---	11.781	0	CBS domain protein	Cytoplasm
PP_1742	---	9.595	4.04E-53	conserved hypothetical protein	Cytoplasmic membrane
PP_2734	*cfa*	6.805	2.93E-244	cyclopropane-fatty-acyl-phospholipid synthase	Cytoplasm
PP_1743	---	6.601	5.75E-276	sodium:solute symporter family protein	Cytoplasmic membrane
PP_2733	---	6.426	2.42E-57	hypothetical protein	-
PP_1000	*argI*	6.269	0	ornithine carbamoyltransferase, catabolic	Cytoplasm
PP_1001	*arcA*	5.546	0	arginine deiminase	Cytoplasm
PP_0999	*arcC*	5.38	0	carbamate kinase	Cytoplasm
PP_0806	---	5.287	0	surface adhesion protein, putative	Extracellular
PP_2735	---	5.275	6.57E-113	conserved hypothetical protein	Cytoplasmic membrane
PP_1408	*phaG*	4.965	0	acyl-transferase	Cytoplasm
PP_0711	---	4.781	2.51E-258	hydrolase, isochorismatase family	-
PP_2730	---	4.711	1.14E-62	lipoprotein, putative	-
PP_0353	---	4.634	9.68E-49	exonuclease	Cytoplasm
PP_1659	---	4.574	1.92E-273	conserved hypothetical protein	Periplasmic
PP_1002	*arcD*	4.458	0	arginine/ornithine antiporter	Cytoplasmic membrane
PP_2732	---	4.225	1.15E-131	conserved hypothetical protein	Cytoplasmic membrane
PP_0805	---	4.152	0	outer membrane efflux protein	Outer membrane
PP_4487	*acsA*	4.109	0	acetyl-coA synthetase	Cytoplasm
PP_1660	---	3.94	1.31E-57	hypothetical protein	Cytoplasmic membrane
PP_2736	---	3.936	3.27E-156	conserved hypothetical protein	-
PP_2361	*csuC*	3.896	0	type 1 pili usher pathway chaperone CsuC	-
PP_4851	---	3.818	0	phosphate starvation-inducible protein, PSIF precursor	-
PP_0804	---	3.757	0	protein secretion ABC efflux system, permease and ATP-binding protein	Cytoplasmic membrane
PP_0803	---	3.7	0	protein secretion ABC efflux system, membrane fusion protein	Cytoplasmic membrane
PP_2705	---	3.669	2.20E-95	hypothetical protein	-
PP_2731	---	3.664	3.74E-27	conserved hypothetical protein	-
PP_2359	---	3.624	0	type 1 pili subunit CsuA/B protein, putative	Extracellular
PP_2360	---	3.539	9.15E-245	type 1 pili subunit CsuA/B protein, putative	Cytoplasmic membrane
PP_0154	---	3.516	8.85E-87	acetyl-CoA hydrolase/transferase	Cytoplasm

				family protein	
PP_2512	*folE-2*	3.51	3.02E-50	GTP cyclohydrolase I	Cytoplasm
PP_0699	---	3.467	3.96E-299	transporter, LysE family	Cytoplasmic membrane
PP_2353	---	3.425	2.22E-13	hypothetical protein	-
PP_1144	---	3.406	5.03E-262	GGDEF domain protein	Cytoplasmic membrane
PP_1661	---	3.398	1.03E-263	dehydrogenase subunit, putative	Extracellular
PP_0103	---	3.393	1.78E-146	cytochrome c oxidase, subunit II	Cytoplasmic membrane
PP_0108	---	3.377	2.27E-15	conserved hypothetical protein	Cytoplasm
PP_1811	*wecB*	3.296	6.83E-50	UDP-N-acetylglucosamine 2-epimerase	Cytoplasm
PP_3504	---	3.282	0	conserved hypothetical protein	-
PP_5008	---	3.273	0	polyhydroxyalkanoate granule-associated protein GA1	Cytoplasm
PP_2729	---	3.259	9.65E-67	hypothetical protein	-
PP_4061	---	3.251	1.14E-16	hypothetical protein	-
PP_4554	---	3.217	9.62E-37	conserved hypothetical protein	-
PP_4409	---	3.188	1.73E-05	site-specific recombinase, phage integrase family	Cytoplasm
PP_4793	---	3.133	1.06E-55	conserved hypothetical protein	-
PP_0109	---	3.096	2.53E-23	membrane protein, putative	Cytoplasm
PP_0105	---	3.092	1.83E-24	cytochrome c oxidase assembly protein	Cytoplasm
PP_3261	---	3.081	0.000272	conserved hypothetical protein	Cytoplasm
PP_2363	*csuE*	3.047	3.08E-62	type 1 pili protein CsuE	-
PP_4555	---	3.026	1.22E-28	conserved hypothetical protein	-
PP_2358	---	3.01	1.68E-209	type 1 pili subunit CsuA/B protein, putative	-
PP_0998	---	3.001	0	conserved hypothetical protein	Cytoplasm
PP_0104	---	2.988	1.42E-65	cytochrome c oxidase, subunit I	Cytoplasmic membrane
PP_2362	*csuD*	2.976	0	type 1 pili usher protein CsuD	Outer membrane
PP_2751	---	2.918	1.35E-05	conserved hypothetical protein	-
PP_3574	---	2.884	8.77E-16	YER057c/YjgF/UK114 family protein	-
PP_3145	---	2.879	0	conserved hypothetical protein	-
PP_4282	---	2.844	5.90E-114	aquaporin Z	Cytoplasmic membrane
PP_0679	---	2.839	0	conserved hypothetical protein	Cytoplasmic membrane
PP_3030	---	2.835	4.75E-10	hypothetical protein	-
PP_0107	---	2.834	4.16E-61	conserved hypothetical protein	Periplasmic
PP_4010	*cspD*	2.822	0	cold-shock protein CspD	Cytoplasm
PP_0763	---	2.813	0	medium-chain-fatty-acid CoA ligase	Cytoplasm
PP_2572	---	2.81	9.57E-51	hypothetical protein	Cytoplasm
PP_2562	---	2.808	1.75E-14	conserved hypothetical protein	-

Table 6. List of downregulated genes in *P. putida* KT2440 Δ*crp vs.* wild-type KT2440 strain in M9-glucose cultures.

Locus	Gene	Fold Change	FDR	Product	Subcellular localization
PP_0424	*crp*	-196.42	0	catabolite gene activator Crp	Cytoplasm
PP_1249	---	-10.579	0	lipoprotein, putative	-
PP_0236	*ssuE*	-8.895	7.23E-180	NADH-dependent FMN reductase	-
PP_0504	*oprG*	-8.305	7.41E-126	outer membrane protein OprG	Outer membrane
PP_3482	---	-5.858	1.93E-07	hypothetical protein	-
PP_4838	*oprC*	-5.563	0	outer membrane copper receptor OprC	Outer membrane
PP_0153	---	-5.547	0	conserved hypothetical protein	-
PP_3330	---	-5.482	1.28E-232	outer membrane ferric siderophore receptor, putative	Outer membrane
PP_3335	---	-5.344	1.99E-172	conserved hypothetical protein	-
PP_3522	---	-5.284	8.50E-08	conserved hypothetical protein	-
PP_5274	---	-5.102	1.65E-90	hypothetical protein	-
PP_1982	*ibpA*	-4.847	0	heat-shock protein IbpA	Cytoplasm
PP_3336	---	-4.76	1.79E-49	hypothetical protein	Cytoplasmic membrane
PP_4837	---	-4.677	0	conserved hypothetical protein	-
PP_4007	*infA*	-4.542	0	translation initiation factor IF-1	Cytoplasm
PP_4626	---	-4.062	1.60E-12	LrgA family protein	Cytoplasmic membrane
PP_3331	---	-4.031	4.75E-79	conserved hypothetical protein	Cytoplasmic membrane
PP_3095	---	-4.016	4.75E-105	chaperone-associated ATPase, putative	Cytoplasm
PP_3100	---	-3.975	2.83E-63	conserved hypothetical protein	Cytoplasm
PP_3405	---	-3.967	1.34E-153	conserved hypothetical protein	Cytoplasmic membrane
PP_4839	---	-3.957	0	membrane protein, putative	Cytoplasmic membrane
PP_4836	---	-3.647	2.10E-293	conserved hypothetical protein	Periplasmic
PP_3748	*glcG*	-3.589	0.000289	glcG protein	-
PP_3099	---	-3.581	7.68E-121	conserved hypothetical protein	Cytoplasm
PP_2655	---	-3.474	1.18E-10	hypothetical protein	-
PP_2070	---	-3.444	0.000267	transcriptional regulator, AraC family	Cytoplasm
PP_5087	*rpmE*	-3.435	0	ribosomal protein L31	Cytoplasm
PP_3091	---	-3.364	3.01E-116	conserved hypothetical protein	Cytoplasmic membrane
PP_3682	---	-3.24	5.70E-15	hypothetical protein	-
PP_1361	*groEL*	-3.234	0	chaperonin, 60 kDa	Cytoplasm
PP_1360	*groES*	-3.223	0	chaperonin, 10 kDa	Cytoplasm
PP_3340	---	-3.214	5.44E-129	TonB-dependent receptor, putative	Outer membrane
PP_4179	*htpG*	-3.189	0	heat shock protein HtpG	Cytoplasm

PP_5392	---	-3.157	9.06E-67	conserved hypothetical protein	Cytoplasm
PP_3092	---	-3.135	9.31E-22	conserved hypothetical protein	Cytoplasm
PP_0318	---	-3.113	2.16E-47	conserved hypothetical protein	-
PP_3093	---	-3.113	1.83E-39	conserved hypothetical protein	Cytoplasm
PP_3332	---	-3.111	1.85E-138	cytochrome c-type protein	-
PP_4178	---	-3.06	5.89E-237	dienelactone hydrolase family protein	-
PP_1820	---	-2.995	4.71E-241	benzoate transport protein	Cytoplasmic membrane
PP_4727	*dnaK*	-2.921	0	dnaK protein	Cytoplasm
PP_2384	---	-2.849	0.000968	conserved hypothetical protein	Cytoplasmic membrane
PP_3088	---	-2.802	2.07E-31	conserved hypothetical protein	-

Table 7. List of upregulated genes in *P. putida* KT2440 Δ*cya*A *vs*. wild-type KT2440 strain in M9-glucose cultures.

Locus	Gene	Fold Change	FDR	Product	Subcellular localization
PP_0354	---	11.151	0	CBS domain protein	Cytoplasm
PP_1742	---	10.488	1.89E-57	conserved hypothetical protein	Cytoplasmic membrane
PP_2734	*cfa*	8.537	0	cyclopropane-fatty-acyl-phospholipid synthase	Cytoplasm
PP_1743	---	8.409	0	sodium:solute symporter family protein	Cytoplasmic membrane
PP_1000	*argI*	7.51	0	ornithine carbamoyltransferase, catabolic	Cytoplasm
PP_0999	*arcC*	7.21	0	carbamate kinase	Cytoplasm
PP_2733	---	5.837	6.71E-47	hypothetical protein	-
PP_1001	*arcA*	5.714	0	arginine deiminase	Cytoplasm
PP_2735	---	5.544	6.09E-117	conserved hypothetical protein	Cytoplasmic membrane
PP_2730	---	5.385	3.50E-75	lipoprotein, putative	-
PP_0806	---	4.806	0	surface adhesion protein, putative	Extracellular
PP_2736	---	4.803	2.85E-215	conserved hypothetical protein	-
PP_4487	*acsA*	4.641	0	acetyl-coA synthetase	Cytoplasm
PP_2189	---	4.596	0	conserved hypothetical protein	-
PP_2135	---	4.551	1.34E-23	hypothetical protein	-
PP_0711	---	4.538	1.88E-223	hydrolase, isochorismatase family	-
PP_0805	---	4.532	0	outer membrane efflux protein	Outer membrane
PP_4851	---	4.493	0	phosphate starvation-inducible protein, PSIF precursor	-
PP_2512	*folE-2*	4.205	4.20E-68	GTP cyclohydrolase I	Cytoplasm
PP_2726	---	4.195	7.32E-05	conserved hypothetical protein	Cytoplasmic membrane
PP_3882	---	4.195	7.31E-05	phage terminase, small subunit, putative	Cytoplasm

PP_1408	*phaG*	4.087	0	acyl-transferase	Cytoplasm
PP_1002	*arcD*	4.063	0	arginine/ornithine antiporter	Cytoplasmic membrane
PP_0353	---	4.024	9.69E-36	exonuclease	Cytoplasm
PP_3030	---	4.005	4.26E-19	hypothetical protein	-
PP_3938	---	4.005	6.28E-05	hypothetical protein	-
PP_2705	---	3.998	2.16E-107	hypothetical protein	-
PP_1660	---	3.996	2.90E-56	hypothetical protein	Cytoplasmic membrane
PP_0103	---	3.939	1.58E-188	cytochrome c oxidase, subunit II	Cytoplasmic membrane
PP_1144	---	3.931	0	GGDEF domain protein	Cytoplasmic membrane
PP_4094	---	3.909	4.84E-10	conserved hypothetical protein	Cytoplasm
PP_3145	---	3.872	0	conserved hypothetical protein	-
PP_1811	*wecB*	3.773	1.32E-62	UDP-N-acetylglucosamine 2-epimerase	Cytoplasm
PP_2732	---	3.733	3.08E-98	conserved hypothetical protein	Cytoplasmic membrane
PP_2729	---	3.651	2.14E-80	hypothetical protein	-
PP_4409	---	3.636	1.61E-06	site-specific recombinase, phage integrase family	Cytoplasm
PP_0699	---	3.609	0	transporter, LysE family	Cytoplasmic membrane
PP_0998	---	3.609	0	conserved hypothetical protein	Cytoplasm
PP_2731	---	3.57	1.82E-24	conserved hypothetical protein	-
PP_2738	---	3.546	3.48E-151	transcriptional regulator, putative	-
PP_2353	---	3.516	2.38E-13	hypothetical protein	-
PP_0803	---	3.511	6.07E-281	protein secretion ABC efflux system, membrane fusion protein	Cytoplasmic membrane
PP_5073	---	3.472	4.56E-18	conserved hypothetical protein TIGR00156	-
PP_5008	---	3.427	0	polyhydroxyalkanoate granule-associated protein GA1	Cytoplasm
PP_4010	*cspD*	3.355	0	cold-shock protein CspD	Cytoplasm
PP_0154	---	3.334	1.40E-73	acetyl-CoA hydrolase/transferase family protein	Cytoplasm
PP_2572	---	3.272	4.68E-68	hypothetical protein	Cytoplasm
PP_1661	---	3.261	1.59E-228	dehydrogenase subunit, putative	Extracellular
PP_4793	---	3.25	2.93E-57	conserved hypothetical protein	-
PP_0028	---	3.231	2.66E-05	conserved hypothetical protein	-
PP_0107	---	3.226	7.42E-78	conserved hypothetical protein	Periplasmic
PP_2737	---	3.216	1.90E-51	oxidoreductase, short-chain dehydrogenase/reductase family	-
PP_2006	---	3.21	0	hypothetical protein	-
PP_2511	---	3.201	1.94E-46	conserved hypothetical protein	-
PP_3676	---	3.188	1.46E-80	hypothetical protein	-
PP_2358	---	3.154	9.40E-221	type 1 pili subunit CsuA/B protein, putative	-
PP_5319	---	3.11	1.20E-185	conserved hypothetical protein	Periplasmic
PP_3512	---	3.089	2.31E-12	conserved hypothetical protein	Cytoplasmic membrane

PP_1609	---	3.072	2.31E-132	hypothetical protein	-
PP_1840	---	3.064	3.93E-35	conserved hypothetical protein	Cytoplasm
PP_4899	---	3.05	1.94E-165	YjeF-related protein	Cytoplasm
PP_0804	---	3.02	0	protein secretion ABC efflux system, permease and ATP-binding protein	Cytoplasmic membrane
PP_1196	---	3.016	3.68E-94	hypothetical protein	-
PP_0396	---	3.011	0	conserved hypothetical protein	Cytoplasm
PP_2853	---	3.005	4.01E-277	conserved hypothetical protein	-
PP_1033	---	2.981	3.31E-225	sulfatase domain protein	Cytoplasmic membrane
PP_4555	---	2.965	6.21E-26	conserved hypothetical protein	-
PP_1947	---	2.961	0.000157	conserved hypothetical protein	-
PP_3611	---	2.954	7.46E-170	hypothetical protein	-
PP_0397	---	2.947	0	conserved hypothetical protein	Cytoplasm
PP_2562	---	2.942	3.30E-15	conserved hypothetical protein	-
PP_0104	---	2.924	4.31E-59	cytochrome c oxidase, subunit I	Cytoplasmic membrane
PP_3223	---	2.904	1.62E-24	ABC transporter, periplasmic binding protein	Periplasmic
PP_3080	*aroF-2*	2.901	3.06E-101	phospho-2-dehydro-3-deoxyheptonate aldolase, class I	Cytoplasm
PP_0679	---	2.888	0	conserved hypothetical protein	Cytoplasmic membrane
PP_3668	---	2.844	0	catalase/peroxidase HPI	Cytoplasm
PP_3469	---	2.838	1.08E-05	hypothetical protein	Extracellular
PP_4240	---	2.83	0.000151	microcin b17 processing protein mcbd, putative	-
PP_3677	---	2.827	6.83E-115	hypothetical protein	Cytoplasm
PP_3678	---	2.82	9.68E-43	hypothetical protein	Cytoplasm
PP_3449	---	2.805	3.32E-29	hypothetical protein	-

Table 8. List of downregulated genes in *P. putida* KT2440 Δ*cya*A *vs*. wild-type KT2440 strain in M9-glucose cultures.

Locus	Gene	Fold Change	FDR	Product	Subcellular localization
PP_5222	*cyaA*	-33.213	0.000252	adenylate cyclase	Cytoplasmic
PP_1249	---	-8.191	1.42E-12	lipoprotein, putative	-
PP_4007	*infA*	-7.756	0	translation initiation factor IF-1	Cytoplasm
PP_0153	---	-7.125	2.79E-13	conserved hypothetical protein	-
PP_1982	*ibpA*	-6.948	0	heat-shock protein IbpA	Cytoplasm
PP_3482	---	-6.283	9.10E-05	hypothetical protein	-
PP_3682	---	-6.154	1.98E-10	hypothetical protein	-
PP_0236	*ssuE*	-6.088	0	NADH-dependent FMN reductase	-
PP_3335	---	-5.557	3.02E-13	conserved hypothetical protein	-
PP_3336	---	-5.392	0	hypothetical protein	Cytoplasmic membrane

PP_0504	*oprG*	-5.383	0	outer membrane protein OprG	Outer membrane
PP_5274	---	-5.374	1.30E-13	hypothetical protein	-
PP_5316	---	-5.369	1.98E-05	hypothetical protein	-
PP_3631	---	-4.855	6.07E-06	conserved hypothetical protein	-
PP_4837	---	-4.76	1.53E-12	conserved hypothetical protein	-
PP_3330	---	-4.7	0	outer membrane ferric siderophore receptor, putative	Outer membrane
PP_3773	---	-4.698	2.02E-05	hypothetical protein	Cytoplasm
PP_3340	---	-4.381	5.49E-14	TonB-dependent receptor, putative	Outer membrane
PP_4839	---	-4.365	4.44E-12	membrane protein, putative	Cytoplasmic membrane
PP_5254	---	-4.224	0.000805	hypothetical protein	-
PP_4836	---	-4.214	0	conserved hypothetical protein	Periplasmic
PP_3100	---	-4.079	0	conserved hypothetical protein	Cytoplasm
PP_3098	---	-3.995	5.38E-13	conserved hypothetical protein	Cytoplasm
PP_4838	*oprC*	-3.983	4.03E-11	outer membrane copper receptor OprC	Outer membrane
PP_4179	*htpG*	-3.954	1.32E-10	heat shock protein HtpG	Cytoplasm
PP_2655	---	-3.933	2.34E-10	hypothetical protein	-
PP_3331	---	-3.894	5.77E-13	conserved hypothetical protein	Cytoplasmic membrane
PP_3853	---	-3.752	0	hypothetical protein	-
PP_2064	---	-3.627	3.85E-08	multidrug efflux RND membrane fusion protein	Cytoplasmic membrane
PP_4233	---	-3.595	0	oxidoreductase, small subunit, putative	Cytoplasm
PP_3405	---	-3.573	0	conserved hypothetical protein	Cytoplasmic membrane
PP_3404	---	-3.565	0	conserved hypothetical protein	Cytoplasm
PP_3780	---	-3.542	0	hypothetical protein	Cytoplasmic membrane
PP_5087	*rpmE*	-3.505	0	ribosomal protein L31	Cytoplasm
PP_1361	*groEL*	-3.504	2.61E-10	chaperonin, 60 kDa	Cytoplasm
PP_3859	---	-3.496	0.000275	conserved hypothetical protein	Cytoplasm
PP_2178	---	-3.454	8.41E-08	glutamine synthetase, putative	Cytoplasm
PP_4626	---	-3.434	5.68E-10	LrgA family protein	Cytoplasmic membrane
PP_4725	*dapB*	-3.31	4.38E-12	dihydrodipicolinate reductase	
PP_4727	*dnaK*	-3.252	0	dnaK protein	Cytoplasm
PP_3211	---	-3.205	8.61E-07	ABC transporter, ATP-binding protein	Cytoplasmic membrane
PP_4178	---	-3.203	0	dienelactone hydrolase family protein	-
PP_1535	---	-3.099	0.000875	methyltransferase, putative	Cytoplasm
PP_0472	*rpmD*	-3.076	0	ribosomal protein L30	Cytoplasm
PP_3465	---	-3.067	0	hypothetical protein	-
PP_3218	---	-2.972	0.000179	monooxygenase, NtaA/SnaA/SoxA family	Cytoplasm
PP_2877	---	-2.954	9.61E-13	transporter, bile acid/Na+ symporter family	Cytoplasmic membrane

PP_4876	*rpsR*	-2.949	2.91E-11	ribosomal protein S18	Cytoplasm
PP_3332	---	-2.927	0	cytochrome c-type protein	-
PP_5001	*hslU*	-2.917	0	heat shock protein HslVU, ATPase subunit HslU	Cytoplasm
PP_3091	---	-2.905	0	conserved hypothetical protein	Cytoplasmic membrane
PP_3092	---	-2.887	0	conserved hypothetical protein	Cytoplasm
PP_3339	---	-2.874	0	conserved hypothetical protein	-
PP_3095	---	-2.87	2.79E-13	chaperone-associated ATPase, putative	Cytoplasm
PP_4728	*grpE*	-2.827	2.91E-11	heat shock protein GrpE	Cytoplasm
PP_0627	---	-2.816	1.55E-06	conserved hypothetical protein	Cytoplasmic membrane
PP_1671	---	-2.809	0	hypothetical protein	-

Summary of the Thesis in Spanish by sections

I. Introducción

La amplia diversidad de microorganismos que se encuentran en la naturaleza se enfrentan con mucha frecuencia a cambios drásticos en la disponibilidad de fuentes de carbono y de nitrógeno. Su supervivencia depende en gran medida de la habilidad con que se adaptan a estos cambios por medio de la regulación de la expresión de genes que codifican para las enzimas y transportadores necesarios para el crecimiento ante los cambios en su medio ambiente. El mecanismo por el cual se lleva a cabo esta expresión genética es muy complejo, y comprende muchas etapas, cada una con una minuciosa regulación. Sin embargo, en bacterias la iniciación de la transcripción es el paso más importante de este proceso.

Los factores de transcripción son proteínas que cumplen un papel fundamental en la iniciación de la transcripción, ya que modulan su activación por medio de la ARN polimerasa, o bien la pueden reprimir al interferir con la actividad de esta enzima. El genoma del microorganismo modelo *Escherichia coli* codifica para alrededor de 300 factores de transcripción, de los cuales diez de ellos (Crp, FNR, IHF, Fis, ArcA, NarL, Lrp, RutR, Cra and Dan) se consideran *reguladores globales*, ya que controlan simultáneamente la transcripción de un gran número de genes. Uno de los más estudiados en procariotas es la proteína receptora de cAMP (Crp o CAP). En *E. coli*, este factor se encuentra formando dímeros que se activan mediante la interacción con el adenosín monofosfato cíclico o cAMP, el cual provoca un cambio estructural en la proteína que le permite interaccionar con secuencias específicas de ADN que se encuentran dentro de los promotores de los genes que ésta regula (esta secuencia es similar al motivo consenso 5'TGTGA-6N-TCACA3'). A pesar de que Crp es una proteína muy abundante en *E. coli*, se ha especializado en controlar las funciones metabólicas en esta bacteria, ya que muchos de los genes que regula codifican para enzimas y proteínas transportadoras de sustratos que son utilizados por la célula como fuentes de carbono. Esto convierte a Crp en uno de los factores principales que median el fenómeno de Represión Catabólica. Este proceso es sumamente importante para la adaptación de los microorganismos a las condiciones cambiantes del medio ambiente en el cual habitan, ya que les permite diferenciar entre fuentes de carbono preferidas (que son metabolizadas rápidamente o que son energéticamente favorables) cuando se encuentran expuestas a más de un nutriente. De esta forma, la regulación de la actividad de Crp en *E.*

coli por medio de la disponibilidad de su molécula efectora (cAMP) le permite activar o reprimir solamente los genes que codifican las enzimas necesarias para utilizar las fuentes de carbono preferenciales.

Como se mencionó anteriormente, la actividad de Crp se encuentra altamente regulada por la concentración intracelular del cAMP, la cual se sintetiza a partir del ATP por la enzima adenilato ciclasa (AC). Existen varias clases de AC, las cuales están presentes en prácticamente todos los dominios de la vida. Entre estas, la CyaA de *E. coli* es probablemente la AC de clase I mejor caracterizada en procariotas. Según el modelo vigente, esta proteína es activada mediante el estado de fosforilación de la proteína $EIIA^{Glc}$ perteneciente al sistema de fosfotransferasa para azúcares dependiente de fosfoenolpiruvato o PTS. Así, cuando la enzima $EIIA^{Glc}$ se encuentra fosforilada, activa a la AC para sintetizar el cAMP. Otro factor importante que regula la concentración intracelular de este nucleótido cíclico es la actividad de las enzimas fosfodiesterasas de cAMP. Estas proteínas catalizan la hidrólisis del fosfato cíclico del cAMP, resultando en la producción del monofostato de adenosina inactivo.

La bacteria del suelo *Pseudomonas putida* es el organismo oportunista por excelencia desde el punto de vista nutricional y además es un modelo de diversidad metabólica entre los organismos capaces de reciclar desechos orgánicos en el medio ambiente aeróbico. Sus muchas características, de las cuales destacan principalmente su gran diversidad metabólica, la capacidad para degradar compuestos xenobióticos y su gran robustez, la han convertido en un organismo modelo para la investigación en bacterias del suelo y para aplicaciones biotecnológicas como biorremediación y biocatálisis. A pesar de que han transcurrido más de 40 años desde el descubrimiento de Crp en *E. coli*, las funciones del dúo cAMP-Crp en *P. putida* no se han llegado a estudiar en profundidad. Su genoma codifica ortólogos singulares de los genes *crp* y *cya*A de *E. coli*. Sin embargo, su función no parece estar relacionada con el metabolismo de fuentes de carbono, y por otra parte parece regular funciones relacionadas con la membrana celular, como la resistencia a antibióticos y antimetabolitos, la motilidad celular y la formación de biopelículas. Adicionalmente, existen evidencias de que el sistema cAMP-Crp puede estar involucrado en la utilización de aminoácidos como fuentes de carbono y nitrógeno en esta bacteria. De este modo, en la

presente Tesis tratamos de esclarecer cual es el mecanismo de activación y regulación de la transcripción del sistema cAMP-Crp en *P. putida*, así como sus funciones fisiológicas.

II. Objetivos

Objetivo General

Investigar los mecanismos de regulación en el sistema de expresión genética basado en el dúo cAMP-Crp, así como las funciones que lleva a cabo.

Objetivos específicos

1. Caracterizar genética y fenotípicamente los genes *crp*, *cya*A y *cpd*A de *P. putida*, así como las proteínas que están codificadas por cada uno de ellos.
2. Detectar y medir los niveles intracelulares del metabolito secundario cAMP en *P. putida.*
3. Purificar y caracterizar bioquímicamente el regulador $Crp_{P.\ putida}$, así como la regulación de su actividad por el cAMP.
4. Detectar los genes que se encuentran regulados directa o indirectamente por el sistema cAMP-Crp de *P. putida*

III. Resultados y Discusión

Capítulo I

En este capítulo se describen los genes que forman parte del sistema de regulación genética basado en el dúo cAMP-Crp en *P. putida*, así como su contexto genómico. Estos genes corresponden a *crp* (que codifica para la proteína de unión a cAMP o Crp), *cya*A (adenilato ciclasa o CyaA) y *cpd*A (fosfodiesterasa de cAMP o CpdA). Mediante curvas de crecimiento, se muestra que la mutación tanto de *crp* como de *cya*A en *P. putida* no tienen ningún efecto perceptible sobre el crecimiento de esta bacteria en varias fuentes de carbono. Por el contrario, la mutación del gene *cpd*A disminuye la tasa de crecimiento en los cultivos crecidos en succinato y glucosa, pero no así en fructosa. Mientras que la función de CpdA puede predecirse mediante homología con otras fosfodiesterasas descritas en la literatura, los

productos de los genes *crp* y *cya*A de *P. putida* se analizaron funcionalmente mediante ensayos de complementación del consumo de maltosa en cepas mutantes de los genes homólogos en *E. coli*. Estos experimentos muestran que la proteína Crp$_{P. putida}$ es realmente funcional y requiere del cAMP para ejercer su actividad regulatoria. Por otra parte, la enzima CyaA$_{P. putida}$ solo fue capaz de complementar la mutación del gene *cya*A en una cepa de *E. coli* hipersensible a bajas concentraciones de cAMP, lo cual supone que la AC de *P. putida* produce niveles muy bajos de cAMP. Estos resultados se confirmaron posteriormente tanto cualitativamente con un biosensor basado en el protozoo *Dictyostelium discoideum* y cuantitativamente mediante HPLC-ESI-MS. Posteriormente, se utilizan fusiones transcripcionales y traduccionales del promotor del gene *cya*A$_{P. putida}$ para demostrar que una de las razones de la baja actividad de la AC de *P. putida* radica en que este gene se transcribe eficientemente, pero su nivel de traducción es muy bajo. Finalmente, se muestra la fusión traduccional del promotor de *crp*$_{P. putida}$ para comprobar que la traducción de este gen se aumenta ligeramente cuando las células crecen en glucosa como única fuente de carbono. La actividad de estos promotores (*cya*A y *crp*) parece ser independiente del dúo regulador cAMP-Crp en *P. putida*.

Capítulo II

Con la finalidad de caracterizar bioquímicamente la proteína Crp$_{P. putida}$, esta se purificó mediante una fusión con la proteína de unión a maltosa (MBP). En el capítulo II se describen los pasos para obtener grandes cantidades de MBP-Crp$_{P. putida}$, que posteriormente se somete a un proceso de proteólisis con trombina y purificación en una resina de fosfocelulosa para obtener la Crp$_{P. putida}$ libre de la *etiqueta* de MBP. A continuación, se utilizan ensayos de ultracentrifugación analítica para determinar el estado de oligomerización de la Crp$_{P. putida}$ pura, que forma dímeros en solución con un tamaño molecular de 51,6 kDa. Estos dímeros sufren un aumento en su dimensión física cuando Crp$_{P. putida}$ interacciona con el cAMP, lo cual se debe a un cambio conformacional en la estructura de la proteína. Este cambio se demuestra asimismo utilizando ensayos de proteólisis parcial en ausencia o presencia de este efector. Con el propósito de calcular los parámetros termodinámicos de la interacción de Crp$_{P. putida}$ con el cAMP, se realizaron ensayos de microcalorimetría de titulación isotérmica. Estos experimentos revelaron que la unión entre el cAMP y Crp$_{P. putida}$ ocurre mediante un proceso exotérmico, con cambios favorables en la

entalpía y en la entropía del sistema. Más importante aún, los ensayos muestran que esta unión se lleva a cabo con una estequiometría de 1 molécula cAMP: 1 dímero $Crp_{P.\ putida}$ y es especialmente fuerte, con una K_D de 45.0 ± 3.4 nM, es decir, una afinidad mucho mayor que la conocida para la proteína homóloga de *E. coli*. La estequiometría tan atípica para este tipo de reguladores transcripcionales se explica mediante un modelo de *cooperatividad negativa* en el cual, la unión del cAMP por uno de los monómeros de $Crp_{P.\ putida}$ disminuye en gran medida la afinidad del cAMP en el bolsillo de unión del monómero restante. $Crp_{P.\ putida}$ también muestra afinidad por el cGMP, pero esta es cerca de 100 veces menor. Finalmente, se utiliza un sistema de transcripción *in vitro* empleando la ARN polimerasa purificada de *P. putida,* junto con sus respectivos ensayos de retardo de la movilidad electroforética para mostrar que $Crp_{P.\ putida}$ es capaz de unir y activar la transcripción del promotor *lac* de *E. coli* únicamente en presencia de cAMP. Esto indica que todas las características básicas de la $Crp_{E.coli}$ necesarias para la iniciación de la transcripción también se encuentran presentes en la Crp de *P. putida.*

Capítulo III

En este capitulo se muestran resultados preeliminares de los experimentos de transcriptómica realizados en las cepas de *P. putida* KT2440, Δ*crp* y Δ*cya*A mediante ultra-secuenciación profunda de ARN (ARN-seq). Estos análisis demuestran que tanto Crp como CyaA regulan muy pocos genes relacionados con funciones metabólicas en *P. putida*. Por el contrario, el dúo regulador cAMP-$Crp_{P.\ putida}$ parece controlar factores relacionados con la respuesta celular a estrés ambiental como por ejemplo GroEL, GroES, DnaK y varias proteínas de choque térmico. Así mismo cAMP-$Crp_{P.\ putida}$ controlaría la expresión de componentes de la envuelta celular, sistemas de secreción de proteínas, los pili tipo I y componentes del citocromo C como respuesta a condiciones estresantes. Es interesante el hecho de que los resultados muestran muchos genes que se ven afectados solamente en alguno de los dos mutantes. Esto abre la posibilidad de que $Crp_{P.\ putida}$ pueda estar actuando también de manera independiente del cAMP, como se ha demostrado para la proteína homóloga Vfr de *P. aeruginosa* o bien, que exista en *P. putida* otro tipo de sistema diferente a Crp que medie la señalización por cAMP.

IV. Conclusiones

El trabajo descrito en esta Tesis permite proponer las siguientes conclusiones:

1. En *P. putida,* el sistema de regulación cAMP-Crp se compone de al menos tres componentes: una proteína de unión a cAMP (Crp), una adenilato ciclasa (CyaA) y una (y posiblemente más de una) fosfodiesterasa de cAMP (CpdA)
2. La proteína Crp$_{P. putida}$ es un regulador transcripcional que se activa mediante regulación alostérica por el cAMP para complementar el fenotipo de metabolismo de maltosa en *E. coli*.
3. La concentración intracelular y extracelular de cAMP producida por la adenilato ciclasa de *P. putida* es muy baja en comparación con las enterobacterias. Estos niveles tan bajos son en parte debido a que el gene que codifica la adenilato ciclasa en este organismo es transcrito eficientemente, pero su traducción es muy baja.
4. La proteína Crp$_{P. putida}$ forma dímeros cuando se encuentra en solución. Estos dímeros sufren un cambio conformacional cuando interaccionan con el cAMP, lo que posiblemente posiciona la proteína en la orientación correcta para reconocer e interaccionar con las correspondientes secuencias de ADN.
5. La proteína Crp$_{P. putida}$ tiene una afinidad extraordinaria por el cAMP, que a su vez determina en gran parte sus funciones en *P. putida*. Esta hipersensibilidad por el cAMP explica por qué las concentración de cAMP en esta bacteria son tan bajas.
6. La proteína Crp$_{P. putida}$ se une al cAMP con una estequiometría de 1 dímero de Crp: 1 molécula cAMP. Esto se explica mediante un modelo de cooperatividad negativa en el cual la unión del cAMP en uno de los monómeros induce un cambio conformacional que reduce en gran medida la afinidad por el cAMP en el bolsillo de unión de ésta molécula en el segundo monómero.
7. El complejo formado por cAMP-Crp$_{P. putida}$ es capaz de unir y activar la transcripción del promotor *lac* (tipo I para Crp).
8. La función reguladora del sistema cAMP-Crp de *P. putida* está relacionada con la respuesta celular al estrés ambiental y con la expresión de componentes de la membrana que modifican el envoltorio bacteriano en esas condiciones. Adicionalmente, este sistema puede regular el metabolismo de ciertos aminoácidos como fuentes de carbono y nitrógeno.